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Innate and adaptive immunity to tumors: IL-12 is required for optimal responses

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We have investigated the importance of endogenously produced IL-12 in innate and adaptive immunity to tumor transplants. The immunogenic lymphoma RMA and its TAP-deficient variant RMA-S were tested for rejection responses by normal and IL-12-deficient mice. IL-12 was crucial for the immunity induced by one immunization with irradiated RMA cells, as well as for *in vivo* priming of a CTL response in mixed lymphocyte tumor cultures against this MHC class I-expressing tumor. The defective *in vivo* response could be overcome by multiple immunizations. In further studies of *in vitro* CTL responses, we found that IL-12 production from either the antigen-pulsed dendritic cells or from host cells was necessary to obtain strong CTL responses. In the complete absence of IL-12, no or only very weak responses could be detected. NK cell-mediated innate resistance, as assessed in non-immunized mice inoculated with a threshold dose of RMA-S cells, also required IL-12. However, NK cells with reduced activity were present in IL-12-deficient mice and contributed to innate resistance, as demonstrated with lower cell dose challenges. In conclusion, IL-12 is required for optimal adaptive and innate responses against tumors.

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1 Introduction

The heterodimeric cytokine IL-12 is produced by antigen-presenting cells (APC) [1] following antigen-specific interactions with T cells through ligation of cell surface CD40 [2, 3]. IL-12 production by APC can also be induced by microbial stimulation [4].

One of the most important effects of IL-12 is to stimulate Th1 responses, by direct effects on T cells and also by induction of IFN-γ production by NK cells [5–8]. IL-12 thereby supports Th1 development which promotes cellular immunity, and not Th2 responses that promote humoral immunity [9, 10]. IL-12 also augments the cytotoxic activity of both NK and cytotoxic T cells [11–17].

The role of IL-12 has been previously studied in various disease models, with either antibody blockade *in vivo* or knockout mice. For example, IL-12 seems to be essential for protective immunity against *Leishmania major* [18, 19], *Mycobacterium tuberculosis* [20] and for dendritic cell (DC)-induced protection against *Chlamydia trachomatis* [21], while it seems to be dispensable for the induction of immunity against *Listeria monocytogenes* [22] and for the generation of Th1 responses during viral infections [23, 24].

Recombinant IL-12 has been shown to have positive effects in immunotherapy of various tumors [25, 26], and we therefore wanted to determine the effects of naturally produced IL-12 in innate as well as adaptive tumor immunity. We used the well-characterized tumor cell line RMA and the TAP-deficient variant RMA-S. The latter has a premature stop codon in the TAP2 genes, which results in defect peptide loading of MHC class I molecules. This leads to low expression of MHC class I on the cell surface, and sensitivity to lysis by NK cells [27]. The rationale behind this choice of experimental model was to study adaptive and innate rejection mechanisms using variants of the same tumor. RMA is relatively NK cell resistant, but highly immunogenic for MHC class I-restricted T cell responses; it is efficiently rejected by C57BL/6 (B6) mice after one immunization with irradiated cells. Conversely, RMA-S is highly NK sensitive and rejected after low-dose inoculation in normal mice. This rejection is abolished by treatment with anti-NK1.1 mAb, and is due to NK cells rather than NK1.1$^+$ T cells, since rejection is also observed in nude mice [28]. In the current study we show that IL-12 is important for *in vivo* rejection of RMA and RMA-S tumor cells as well as for T cell- and NK cell-mediated cytotoxicity *in vitro*. We also

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Abbreviations: **DC**: Dendritic cell **B6**: C57BL/6

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show that DC-mediated immunization against a defined MHC class I-restricted synthetic peptide is IL-12 dependent.

2 Results

2.1 IL-12 is required for optimal rejection of RMA tumor cells and *in vitro* cytotoxicity against RMA

We first investigated the role for IL-12 in adaptive immunity against RMA tumor cells. B6 and IL-12$\alpha^{-/-}$ mice were immunized with irradiated RMA tumor cells and challenged with live tumor cells 1 week after the last immunization. Mice were given one or three immunizations. Following one immunization, B6 but not IL-12$\alpha^{-/-}$ mice were able to completely reject the inoculated RMA cells. However, after three immunizations both B6 mice and IL-12$\alpha^{-/-}$ rejected the inoculated cells (Table 1).

To test whether the results from the *in vivo* experiments could be correlated with the generation of a cytotoxic response, spleen cells from immunized mice were restimulated *in vitro* and tested for CTL activity against RMA tumor cells. The restimulated B6 cultures were consistently more efficient at killing the tumor target than cultures from IL-12$\alpha^{-/-}$ mice, even after three immunizations (Fig. 1). Mice immunized only once did not yield any CTL responses that could be detected *in vitro* by the methods used in this study.

2.2 Cytotoxicity following DC-mediated immunization with a synthetic peptide is IL-12 dependent

One possible explanation for our observations was that priming of CTL is dependent on IL-12 produced by the APC. This question was addressed by immunizing B6 and IL-12$\alpha^{-/-}$ mice with a synthetic peptide (corresponding to LCMV GP33–41) loaded on bone marrow-derived DC from B6 or IL-12$\alpha^{-/-}$ mice. Spleen cells were restimulated *in vitro* with the synthetic peptide and tested for the presence of CTL against peptide-loaded target cells. The generation of the CTL response was IL-12 dependent since immunization of IL-12$\alpha^{-/-}$ mice with DC from IL-12$\alpha^{-/-}$ mice resulted in no or only very weak CTL responses (Fig. 2). Immunization of B6 mice with IL-12-deficient DC or immunization of IL-12-deficient mice with B6 DC resulted in strong CTL responses. This shows that the IL-12 necessary for DC-mediated immunization could be provided by the antigen-pulsed APC or by host cells.

Table 1. Optimal rejection of RMA tumor cells requires IL-12

Mice		Number of mice with tumors	% of mice with tumors
B6[a]		7/8	88
IL-12$\alpha^{-/-}$[a]		8/8	100
B6	1 immunization[a]	0/8	0
IL-12$\alpha^{-/-}$	1 immunization[a]	7/8	88
B6	3 immunizations[b]	0/11	0
IL-12$\alpha^{-/-}$	3 immunizations[b]	1/11	9

[a] Unimmunized mice and mice immunized ince were inoculated with 500 live RMA tumor cells

[b] Mice immunized three times were inoculated with 10^4 live RMA tumor cells. 100% of the unimmunized controls that received this dose developed tumors.

2.3 Optimal rejection of TAP-deficient RMA-S tumor cells requires IL-12

Since IL-12 is an important NK cell stimulator, we also investigated the possible role of IL-12 in natural immunity against TAP-deficient RMA-S cells by inoculation of different numbers of live RMA-S cells into B6 and IL-12$\alpha^{-/-}$ mice. IL-12$\alpha^{-/-}$ had a defective natural resistance to RMA-S cells. This was most obvious at the threshold

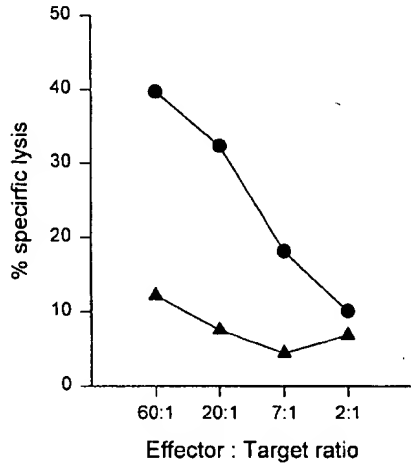

Fig. 1. Spleen cells from B6 mice immunized against RMA show higher cytotoxic activity *in vitro* then cells from IL-12$\alpha^{-/-}$ mice. Mice were immunized with irradiated RMA tumor cells. Spleen cells from B6 (●) and IL-12$\alpha^{-/-}$ (▲) were restimulated *in vitro* and tested for lysis of RMA cells. Lysis of B6 Con A blasts or RMA-S was always below 5%. One representative experiment of three is shown.

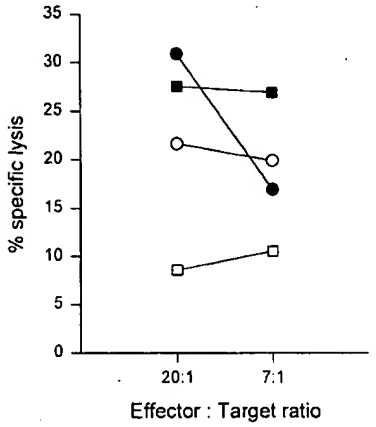

Fig. 2. DC-mediated immunization against synthetic peptides is IL-12 dependent. B6 and IL-12α$^{-/-}$ mice were immunized with peptide-loaded, bone marrow-derived DC from B6 or IL-12α$^{-/-}$. The following combinations were tested; B6 DC to B6 mice (●), B6 DC to IL-12α$^{-/-}$ mice (○), IL-12α$^{-/-}$ DC to B6 mice (■) and IL-12α$^{-/-}$ DC to IL-12α$^{-/-}$ mice (□). All effector cells were tested against RMA-S with and without the relevant peptide. Lysis of RMA-S without peptide was always below 5%. One experiment of three is shown.

dose of 10^6 tumor cells; the tumor cells were completely rejected by all B6 mice, but grew progressively in half of the IL-12$^{-/-}$ mice (Table 2). Even at the higher dose of 5×10^6 cells, which grew in all animals, there was a clear delay in latency period before tumor appearance (*e.g.* 100% of the inoculated mice developed palpable tumors

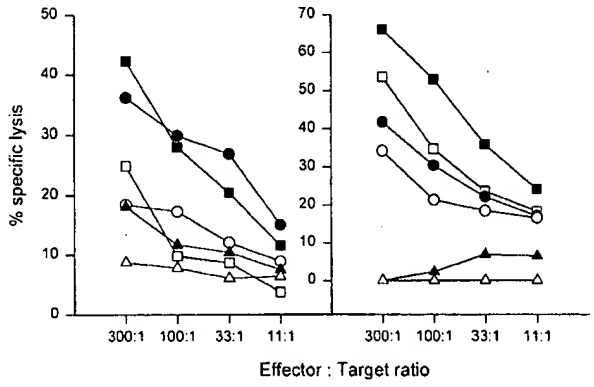

Fig. 3. NK-mediated cytotoxicity against RMA-S and YAC-1 is augmented by IL-12. Following activation *in vivo* by tilorone, spleen cells from B6 and IL-12α$^{-/-}$ mice were tested for lysis against YAC-1, RMA-S and RMA [B6 against YAC-1 (■), IL-12α$^{-/-}$ against YAC-1 (□), B6 against RMA-S (●), IL-12α–/– against RMA-S (○), B6 against RMA (▲) and IL-12α$^{-/-}$ against RMA (△)]. Two experiments of five are shown.

Table 2. Optimal rejection of TAP-deficient RMA-S tumor cells requires IL-12

Mice	Inoculated cells	Number of mice with tumors[a)]	% of mice with tumors
B6	10^5	0/22	0
IL-12α$^{-/-}$	10^5	0/22	0
B6	10^6	0/22	0
IL-12α$^{-/-}$	10^6	11/21	52
B6	5×10^6	8/8	100
IL-12α$^{-/-}$	5×10^6	8/8	100
NK1.1-depleted mice			
B6	10^5	6/6	100
IL-12α$^{-/-}$	10^5	6/6	100
B6	10^6	6/6	100
IL-12α$^{-/-}$	10^6	6/6	100

[a)] Each value represents at least two experiments and the first four groups were included in all experiments.

by day 8 in IL-12α$^{-/-}$ mice as compared with day 12 in B6 mice, data not included). However, both B6 and IL-12α$^{-/-}$ mice were able to completely reject 10^5 cells. Depletion of NK1.1$^+$ cells before inoculation of live tumor cells confirmed that NK cells were responsible for the rejection (Table 2). This treatment not only abolished the difference between B6 and IL-12α$^{-/-}$ mice at the threshold dose of 10^6 tumor cells, but also rendered both types of mice susceptible to the lower dose of the tumor (10^5 cells). We conclude that IL-12 is important for optimal innate immunity, but not absolutely essential for responses to weaker challenges.

To test whether a similar difference could be detected *in vitro* we performed NK cell cytotoxicity assays with cells from mice treated with the IFN-inducer tilorone, known to induce NK activity. Cells from B6 mice consistently killed YAC-1 and RMA-S more efficiently than did cells from IL-12α$^{-/-}$ mice (Fig. 3). However, there was often also substantial activity mediated by the IL-12α$^{-/-}$ cells. In terms of effector cell dilution, the difference varied between three- and tenfold for YAC –1 target cells.

3 Discussion

The results in this study indicate that IL-12 has an important role in the immune response against tumors. One interesting finding is that rejection of RMA tumor cells is IL-12 dependent in mice that have received one immuni-

zation, while mice that have received three immunizations are able to reject high numbers of RMA cells. Although it is still possible to detect differences in cytotoxic activity *in vitro* after three immunizations, this indicates that IL-12 may have its primary role during the early phase of the immune response. This is in agreement with previous findings in a study of the immune response against *L. monocytogenes* by Oxenius et al. [24] showing that IL-12-deficient mice were unable to mount a normal early response against *Listeria*, although they could clear the infection at a later stage.

Through further analysis of the role of IL-12 in CTL responses, we demonstrated that IL-12 is also required in responses induced by antigen-pulsed DC, and that DC-derived IL-12 itself is sufficient to restore the response. However, it was also possible to generate good CTL responses by immunizing B6 mice with IL-12-deficient DC. There are different possible interpretations of this observation. For example, DC-derived IL-12 may still be required, but the inoculated DC may be engulfed and presented by host APC. Another possibility is that MHC class I-peptide complexes are transferred from the inoculated cells to host APC, which are able to produce IL-12. It has previously been reported that MHC class II molecules can be transferred from APC to T cells [29]. Alternatively, neighboring cells may provide IL-12 required for optimal responses.

As to innate immunity, we observed a difference in the ability to reject TAP-deficient RMA-S between wild-type and IL-12-deficient mice. However, this was only observed with comparatively high tumor cell doses. At a lower dose, both B6 and IL-12$\alpha^{-/-}$ mice rejected RMA-S cells efficiently in an NK-dependent manner. These *in vivo* observations correlated well with *in vitro* experiments by others [30] and by us in this study: IL-12$\alpha^{-/-}$ mice posses active NK cells, but the levels are not as high as those observed with wild-type mice. One may ask if this difference observed in cytotoxicity can account for the differences in capacity to completely reject a tumor graft, as observed with the dose of 10^6 cells. It may be that IL-12 affects additional factors in the NK response *in vivo*, such as migration or recruitment of NK cells to the tumor site. However, Smyth et al. [31] observed no difference between IL-12$^{-/-}$ and B6 mice with respect to early NK infiltration in ascites RMA-S tumors; TNF-α appeared to be the important cytokine in this situation. Smyth et al. [31] further concluded that IL-12 did not influence NK cell-mediated rejection at all. Our results are not consistent with this notion; IL-12 appears to be critical, at least for rejection of higher tumor cell doses.

In conclusion, IL-12 seems to be important for immune responses against the tumor used in our model. IL-12 was also important for the generation of CTL responses against a synthetic peptide, suggesting that IL-12 may also be important for the generation of immune responses against other antigens. The observations in our model systems suggest that, although IL-12 is not absolutely required for adaptive or innate immunoreactivity, it seems to be essential for an optimal, early response. It should be stressed that our model systems has been chosen and titrated to include at least one observation point at which the response is as strong as possible (three immunizations in the test of adaptive immunity and the low dose 10^5 in the test of innate immunity). The influence of IL-12 may, therefore, be dramatic in pathophysiological or in immunological responses against weak antigens, which may correspond better to the situations in which we observed substantial defects in IL-12$\alpha^{-/-}$ mice (one immunization in adaptive immunity and the threshold dose of 10^6 in innate immunity). This is importance for different aspects of immunotherapy, and may also have implications for other immunological problems. One such example is immunodominance, whereby injection of IL-12 altered dominance patterns after immunization with a mixture of synthetic peptides [32]. Competition for cytokines and possibly IL-12 has also been suggested as a mechanism for immunodominance of minor histocompatibility antigens [33]. We have previously demonstrated that immunodominance can be broken by immunization with DC, which might be an effect of IL-12 production [34–36].

In the future it will be important to study the detailed role of IL-12 in induction of cellular immunity and the possible roles for IL-12 in immunotherapy against tumors.

4 Materials and methods

4.1 Mice and cell lines

The inbred mouse strain C57BL/6By (B6) and IL-12$\alpha^{-/-}$ mice were purchased (Jackson laboratory, Bar Harbor, ME) and further bred and maintained at the Microbiology and Tumor Biology Center, Karolinska Institutet, Sweden. Tumor cell lines RMA and the TAP2-deficient variant RMA-S was maintained at 37°C and 5% CO_2 in RPMI 1640 tissue culture medium supplemented with 5% FCS, 50 μg streptomycin/ml, 100 μg penicillin/ml and 2 mM L-glutamine.

4.2 *In vivo* NK cell depletion

One day before inoculation of tumor cells, each mouse received an intraperitoneal injection of 50 μg anti-NK1.1 mAb (PK136; mouse IgG2a).

4.3 Tumor outgrowth experiments

B6 and IL-12$\alpha^{-/-}$ were inoculated s.c. with titrated numbers of RMA or RMA-S tumor cells. Tumor outgrowth was followed for 8 weeks after inoculation.

4.4 Generation of bone marrow-derived DC

Bone marrow-derived DC were obtained using the protocol of Inaba et al. [37] with minor modifications. Bone marrow cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% supernatant from the GM-CSF secreting cell line X63 (a gift from Dr. D. Gray through Dr. C. Watts, University of Dundee, Dundee, GB) and 20% FCS. The culture medium was replaced every 3rd day, and the cells were replated on day 7.

4.5 Synthetic peptide

The synthetic H-2D^b-presented peptide GP33–41 KAVYNFATM [38, 39] from lymphocytic choriomeningitis virus (LCMV) was synthesized using solid-phase Fmoc chemistry.

4.6 *In vivo* priming

B6 or IL-12$\alpha^{-/-}$ mice were immunized with 2×10^6 irradiated RMA tumor cells or with one s.c. inoculation of 5×10^5 live bone marrow-derived DC. Before inoculation DC were incubated at 37°C with 30 μM of the synthetic peptide in serum-free DMEM and washed twice.

4.7 *In vitro* restimulation

Single-cell suspensions of spleens from immunized or non-immunized mice were prepared; 25×10^6 effector cells were incubated with 2×10^6 irradiated (100 Gy) tumor cells, or 10×10^6 irradiated (20 Gy) B6 spleen cells and 1 μM of the synthetic peptide, in 15 ml of α-MEM medium supplemented with penicillin-streptomycin, 10% FCS, 3×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 2 mM L-glutamine at 37°C and 10% CO_2 for 5 days.

4.8 Activation of NK cells with tilorone

One day before the cytotoxicity assay mice were given 2 mg tilorone analogue R10,874DA (Sigma Chemical Co., St. Louis, MO) diluted in 0.2 ml PBS per os.

4.9 *In vitro* cytotoxicity assay

Target cells were labeled with ^{51}Cr and resuspended in cell culture medium. Target cells (5×10^3) were added to each well followed by addition of effector cells. The cells were incubated for 4 h at 37°C and supernatants were harvested. Radioactivity was measured in a Pharmacia-LKB γ-counter, and specific lysis was calculated [(cpm released with effector cells–cpm released without effector cells)/(cpm released by detergent-cpm released without effector cells)]×100. Experiments with more than 30 % spontaneous lysis was discarded.

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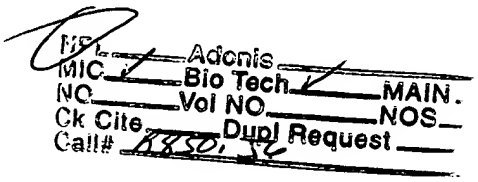

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Recognition of the Major Histocompatibility Complex Restriction Element Modulates CD8+ T Cell Specificity and Compensates for Loss of T Cell Receptor Contacts with the Specific Peptide

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Summary

Triggering of a T cell requires interaction between its specific receptor (TCR) and a peptide antigen presented by a self–major histocompatibility complex (MHC) molecule. TCR recognition of self-MHC by itself falls below the threshold of detection in most systems due to low affinity. To study this interaction, we have used a read-out system in which antigen-specific effector T cells are confronted with targets expressing high levels of MHC compared with the selecting and priming environment. More specifically, the system is based on $CD8^+$ T cells selected in an environment with subnormal levels of MHC class I in the absence of β_2-microglobulin. We observe that the MHC restriction element can trigger viral peptide-specific T cells independently of the peptide ligand, provided there is an increase in self-MHC density. Peptide-independent triggering required at least four times the natural in vivo level of MHC expression. Furthermore, recognition of the restriction element at expression levels below this threshold was still enough to compensate for lack of affinity to peptides carrying alanine substitutions in major TCR contact residues. Thus, the specificity in TCR recognition and T cell activation is fine tuned by the avidity for self-MHC, and TCR avidities for peptide and MHC may substitute for each other. These results demonstrate a functional role for TCR avidity for self-MHC in tuning of T cell specificity, and support a role for cross-reactivity on "self" during T cell selection and activation.

Key words: T cell • specificity • major histocompatibility complex restriction • major histocompatibility complex class I • peptide

Triggering of a $CD8^+$ T cell requires TCR interaction with a specific peptide bound to an MHC class I molecule. The requirement for the selecting MHC molecule in recognition of antigen is known as MHC restriction (1, 2). Although interactions between TCR and the restriction element have been detected only in the presence of specific peptides, it is believed that thymic positive selection imposes a low self-MHC affinity onto the peripheral T cell pool (3–6). Early studies of TCR–MHC interactions involved analyses of MHC mutants for their ability to disrupt T cell reactivity (7–9). Mutations in the α-helical regions in the α1/α2 domains of the MHC molecule were found to perturb recognition of specific antigen, indicating that the TCR physically contacts the α-helices of MHC molecules. However, measurements of affinity between TCR and peptide–MHC complexes have revealed relatively low affinities for MHC in complex with agonist peptides and slightly lower affinities for MHC in complex with antagonist peptides, whereas no detectable affinities have been found for MHC in complex with unrelated peptides (10–13). Thus, the idea of TCR affinity to self-MHC in the absence of specific antigen has been hard to support biochemically.

Recently, crystallographic studies have shown a physical interaction between TCR and MHC in the presence of specific peptide (14, 15). Of the total surface area contacted by the TCR in the MHC complex, approximately one third is provided by the peptide while MHC α1/α2 domains contribute two thirds of the contact area. Based on alanine scanning mutagenesis of the 2C TCR, it has been estimated that almost two thirds of the binding energy in antigen-specific binding is attributable to contacts with the α-helices (16). Does the interaction between TCR and MHC as observed in crystallographic and biochemical studies also contribute to the avidity required for T cell triggering, and can it be detected in the absence of the specific peptide? Further, how does this interaction influence T cell specificity for the antigenic peptide? The answers to

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these questions are of major importance for the understanding of several aspects of physiological T cell function, including MHC restriction (1, 2), thymic selection (17), and maintenance of T cell memory (18). Interestingly, recent evidence indicates that T cells require interaction with endogenous peptide–MHC complexes for long-term survival, suggesting continuous cross-recognition of self for maintenance of immunological memory (19–21).

T cell avidity for self has been demonstrated in functional assays. CTLs triggered by allogeneic MHC can specifically kill bystander cells of self-MHC haplotype, indicating that self-MHC can mediate binding to CTLs that are efficiently triggered by their antigen, although it cannot trigger CTLs directly (22). Second, mice with low MHC class I expression select T cells adapted to that environment, whose avidity to self-MHC is revealed when assessed in the context of normal MHC class I levels. For example, CTLs generated against allogeneic cells in such mice specifically cross-react with cells expressing high levels of self-MHC class I (23–25). Similarly, T cell hybridomas derived from CD3ζ/η-deficient mice expressing very low levels of cell surface TCR also react against cells expressing self-MHC class I molecules, once a normal level of TCR expression has been restored by CD3ζ/η transfection (26). Thus, in systems where either the MHC or the TCR are expressed at subnormal levels, it is possible to generate T cells for the study of TCR affinity/avidity for self-MHC, which are not present in normal mice with high MHC expression due to negative selection.

To study the role of self-MHC avidity in MHC-restricted CTLs, we immunized β_2-microglobulin–deficient ($\beta_2m^{-/-}$)[1] mice, which have low expression of conformed MHC class I (27, 28), with the H-2D^b–restricted immunodominant lymphocytic choriomeningitis virus (LCMV) GP 33-41 (GP33) peptide. We obtained CD8$^+$ CTLs which were specific for the GP33 epitope when loaded onto cells with low MHC class I expression (MHC^{low}). However, unlike GP33-specific C57Bl/6 (B6) CTLs, these CTLs also killed cells expressing high levels of self-MHC class I (MHC^{high}) in the absence of specific peptide. Further experiments revealed a dual specificity of these CTLs: peptide specificity against MHC^{low} targets, and peptide-independent specificity for the restriction element when tested against MHC^{high} targets. By mAb blocking of the MHC restriction element, we found that peptide-independent triggering of a CTL clone with high avidity for self-MHC required at least four times the in vivo expression of MHC. Most interestingly, at lower MHC expression an increased recognition of self-MHC could compensate for lack of TCR interaction with the specific peptide, as evaluated with peptides carrying alanine substitutions in major TCR contact residues. These results indicate that TCR avidity for the MHC molecule functionally contributes to T cell specificity, and that TCR affinities for MHC and peptide are interchangeable. Our results have important implications for the understanding of T cell specificity and the role of self-MHC in peripheral T cell function.

[1]*Abbreviations used in this paper:* β_2m, β_2-microglobulin; B6, C57Bl/6; LCMV, lymphocytic choriomeningitis virus; TAP, transporter associated with antigen processing.

Materials and Methods

Mice. B6, transporter associated with antigen processing (TAP)1$^{-/-}$ (29), $\beta_2m^{-/-}$ (30, 31), and TAP1/$\beta_2m^{-/-}$ (32) mice of the H-2^b haplotype were bred at the Microbiology and Tumor Biology Center, Karolinska Institute. Animal care was in accordance with institutional guidelines. TAP1$^{-/-}$, $\beta_2m^{-/-}$, and TAP1/$\beta_2m^{-/-}$ mice used were back-crossed to B6 background 6, 10, and 7 times, respectively. For thymectomy experiments, $\beta_2m^{-/-}$ mice were 735 rad–irradiated and thymectomized or sham-thymectomized, and reconstituted with $\beta_2m^{-/-}$ fetal liver hematopoietic cells.

Cell Lines. RMA is a subline of the Rauscher virus–induced B6 lymphoma RBL-5, and RMA-S is a TAP2-deficient variant of RMA (33). T2 is a hybrid between the two human cell lines 0.174 and CEM, and has an antigen-processing deficiency due to a deletion on the MHC class II region including the TAP1 and TAP2 genes. T2D^b is an H-2D^b transfectant of T2. All cell lines were maintained at 37°C and 5% CO_2 in RPMI 1640 tissue culture medium supplemented with 5% FCS, 50 μg/ml streptomycin, 100 μg/ml penicillin, and 2 mM L-glutamine. Con A–activated blasts were generated by culturing erythrocyte-depleted splenocytes in 5 μg/ml of Con A for 2 d in tissue culture medium as described above with 10% FCS.

Synthetic Peptides. The following synthetic H-2D^b–presented peptides were synthesized using solid phase F-moc chemistry: LCMV GP33 KAVYNFATM (34, 35); LCMV GP 33-4A (GP33-4A) KAVANFATM; LCMV GP 33-34A (GP33-34A) KAAANFATM; LCMV GP 33-348A (GP33-348A) KAAANFAAM; influenza PR8 NP 366-374 (NP366) ASNENMETM (36); and *Yersinia enterocolitica* Yop51 249-257 (Yop249) IQVGNTRTI (37).

Generation of LCMV GP33-specific CTL Cultures and Clones. GP33-specific CD8$^+$ CTLs were elicited in B6 and $\beta_2m^{-/-}$ mice by peptide immunization (38). 100 μg peptide was dissolved in distilled water and mixed with IFA in a 1:1 ratio by sonication, then injected subcutaneously in the base of the tail. 12 d after immunization, 25 × 10^6 immune spleen cells were cocultured with 25 × 10^6 2,000 rad–irradiated B6 or $\beta_2m^{-/-}$ splenocytes in the presence of 0.05 μM peptide in 12 ml complete medium (RPMI 1640 supplemented with 10% FCS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 5 × 10^{-5} M 2-ME, 2 mM L-glutamine, and 50 μg/ml streptomycin, 100 μg/ml penicillin) at 37°C and 5% CO_2. 6–7 d later, these cells were used as effector cells in a ^{51}Cr-release assay. CTL clones were generated by limiting dilution cloning. Long-term CTL clones and lines were maintained in complete medium (see above) based on MEM-α and further supplemented with Hepes buffer and 20 IU/ml IL-2. CTL clones and lines were restimulated in 12-d intervals with 2,000 rad–irradiated splenocytes in the presence of 0.05 μM peptide.

mAbs and FACS® Analysis. B22-249.1 is a mouse mAb which binds to a conformation-dependent epitope on the α1 domain of the H-2D^b molecule, and Y3 is a mAb which binds to conformed H-2K^b molecules. For flow cytometry with these mAbs, cells were incubated with mAb for 30 min on ice, washed, and then stained with goat anti–mouse Oregon Green conjugate

(Molecular Probes) on ice for 30 min. For measurement of CD8 and TCR expression, CTLs were stained for 30 min with the FITC-conjugated anti-CD8α mAb 53-6.7 (PharMingen) and the PE-conjugated anti-TCR-α/β mAb H57-597 (PharMingen), respectively. After washing, analysis was performed using a FACScan® (Becton Dickinson) with CellQuest software.

CTL Assay, Cold Target Competition, and CTL Blocking with mAb. CTL activity was measured in a standard ^{51}Cr-release assay. In brief, peptide-coated target cells were prepared by incubating cells with indicated concentrations of peptide for 1 h at 37°C. Coated cells were labeled with 10 μl 10 mCi/ml ^{51}Cr for 1 h at 37°C. Titrated amounts of effector cells were incubated with 3×10^3 ^{51}Cr-labeled target cells for 4 h at 37°C, 5% CO_2. After incubation, released radioactivity was measured and specific lysis was calculated according to the formula: % specific release = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Cold temperature-treated target cells were generated through culture of RMA-S at 26°C for 12 h (39). For cold target competition experiments, effector T cells and unlabeled (cold) competitor cells were mixed and preincubated at 37°C for 30 min before addition of labeled (hot) target cells. The assay was then run as a standard ^{51}Cr-release assay for 4 h. For blocking of CTL recognition with the B22-249.1 mAb, titrated amounts of mAb were included in the CTL assay medium.

Results

Expression of Properly Conformed H-2D^b in $\beta_2m^{-/-}$ Cells Is TAP Dependent. We first compared the levels of properly conformed H-2D^b molecules on the cell surface of $\beta_2m^{-/-}$, TAP1/$\beta_2m^{-/-}$, and B6 (TAP1/$\beta_2m^{+/+}$) control cells stained with an mAb directed against H-2D^b (B22-249.1) or H-2K^b (Y3) conformation-sensitive epitopes. A substantial level of conformed cell surface H-2D^b molecules was found on $\beta_2m^{-/-}$ cells, whereas conformed H-2K^b was detected at lower levels, in line with previously published results (27, 28; Fig. 1). This is compatible with H-2D^b being more independent of β_2m during folding and transport of MHC class I free heavy chains (27, 28). On Con A blasts deficient for both β_2m and TAP1, the staining with B22-249.1 was virtually at background levels, which implies that the pool of conformed H-2D^b molecules on $\beta_2m^{-/-}$ cells is dependent on a functional TAP complex.

$\beta_2m^{-/-}$ Mice Have Thymus-dependent CD8$^+$ T Cells that Mount an H-2D^b-restricted and Peptide-specific CTL Response against the LCMV GP33 Epitope. B6 and $\beta_2m^{-/-}$ mice were immunized with antigenic peptides restricted to either H-2D^b or H-2K^b. All tested peptides primed responses in B6 mice (data not shown), whereas only one, the H-2D^b–restricted LCMV GP33, primed a response in $\beta_2m^{-/-}$ mice. This is in line with the low but significant levels of folded H-2D^b molecules on the surface of $\beta_2m^{-/-}$ cells (27; Fig. 1). CTLs from both B6 and $\beta_2m^{-/-}$ mice primed with the GP33 peptide killed RMA-S cells pulsed with the GP33 peptide used for priming but not a control influenza NP366 peptide (Table I). CTL responses were mediated by CD8$^+$ cells as determined by mAb- and complement-mediated depletion of effector populations in vitro (data not shown). FACS® analysis of both polyclonal bulk populations and clones confirmed the TCR$^+$CD8$^+$ phenotype of

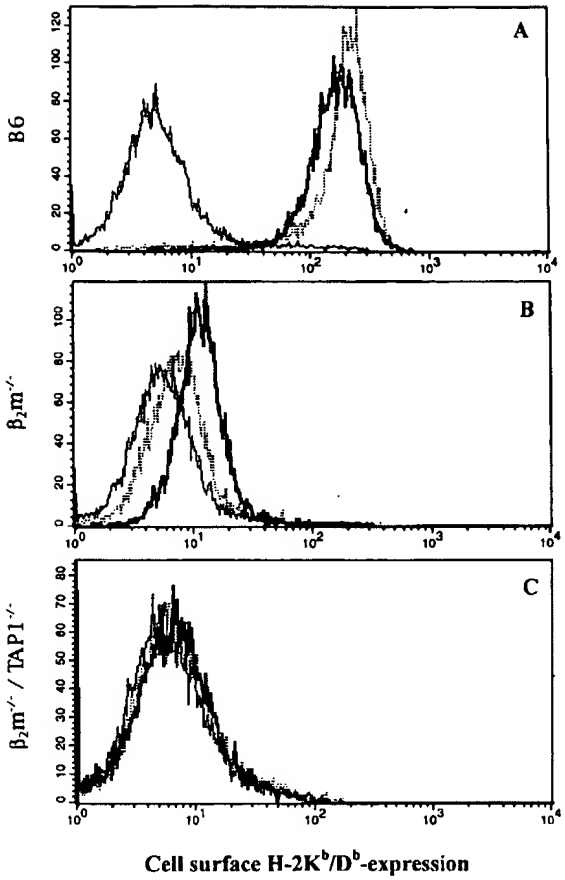

Figure 1. H-2D^b expression in the absence of β_2m is TAP dependent. FACS® analysis of H-2D^b and H-2K^b expression on Con A–activated blasts from B6 (A), $\beta_2m^{-/-}$ (B), and TAP1/$\beta_2m^{-/-}$ (C) mice using the B22-249.1 and Y3 mAbs, respectively. Bold and dotted lines represent H-2D^b and H-2K^b expression, respectively. Solid thin line represents staining with secondary antibody only.

the responding CTLs in $\beta_2m^{-/-}$ as well as B6 mice (Fig. 2). Cell surface expression levels of both TCR and CD8 were similar in CTLs from $\beta_2m^{-/-}$ and B6 mice. However, a marginal increase in CD8 expression levels was detected in some $\beta_2m^{-/-}$ CTLs compared with B6 CTLs. This increase may be due to the low levels of H-2D^b expressed during selection and priming in the absence of β_2m. However, lysis performed by $\beta_2m^{-/-}$ CTLs did not show increased dependency on CD8 compared with B6 CTLs, as determined by mAb blocking of CD8 (data not shown).

No priming of CTLs was observed in thymectomized $\beta_2m^{-/-}$ mice that had been irradiated and reconstituted with fetal liver (Table I). Control mice (receiving irradiation and fetal liver reconstitution without thymectomy) generated a normal antipeptide CTL response, showing that the responding CD8$^+$ T cell population in $\beta_2m^{-/-}$ mice was dependent on the thymus for development. In addition, a normal antipeptide response was also found in mice that had been thymectomized but not irradiated, showing that the thymus was not necessary during the

Table I. *Development of Peptide-specific $\beta_2m^{-/-}$ CTLs Is Thymus Dependent*

Treatment	Target	E:T	Responder mouse strain				
			$\beta_2m^{-/-}$*	B6	$\beta_2m^{-/-}$	$\beta_2m^{-/-}$	$\beta_2m^{-/-}$
Thymectomy‡			−	−	−	+	+
Irradiation and reconstitution			−	−	+	+	−
	RMA-S plus GP33	45:1	49	79	47	1	41
		15:1	38	55	37	1	20
		5:1	23	34	15	0	12
	RMA-S plus NP366	45:1	3	1	−	−	−
		15:1	2	0	−	−	−
		5:1	2	0	−	−	−
	RMA-S, no peptide	45:1	3	2	0	0	2
		15:1	3	1	2	0	0
		5:1	0	0	4	0	3

*Mice immunized with synthetic LCMV GP33 peptide were restimulated in vitro and tested in a ^{51}Cr-release assay against RMA-S cells coated with the indicated peptides as described in Materials and Methods.
‡$\beta_2m^{-/-}$ mice were thymectomized or sham-thymectomized, 735 rad-irradiated, and reconstituted with fetal liver hematopoietic cells as indicated.

priming of the response (Table I). The latter control indicated that the generated CTLs were not positively selected in the thymus in response to the peptide injected for immunization. We conclude that $\beta_2m^{-/-}$ mice have a peripheral pool of $CD8^+$ T cells which are thymus dependent, H-$2D^b$ restricted, and peptide specific despite expressing very low levels of H-$2D^b$ molecules, corresponding to a few percent of those expressed by B6 mice.

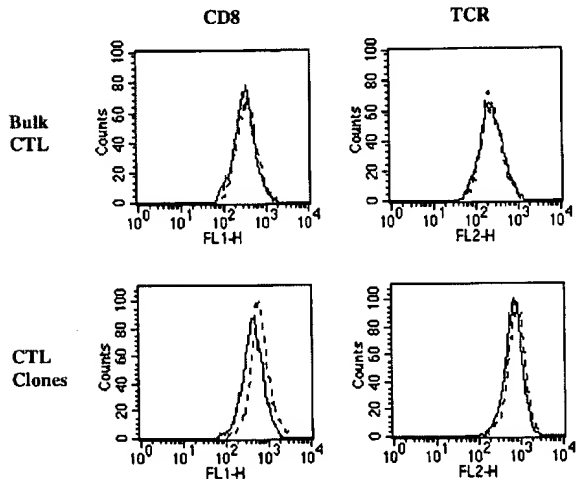

Figure 2. TCR and CD8 coreceptor expression in $\beta_2m^{-/-}$ and B6 CTLs. FACS® analysis of TCR-α/β and CD8α in GP33-specific CTLs, using the PE-conjugated anti-TCR-α/β mAb H57-597 and the FITC-conjugated anti-CD8α mAb 53-6.7 (PharMingen), respectively. Solid lines represent B6 CTLs; dotted lines represent $\beta_2m^{-/-}$ CTLs. In staining of CTL clones, the representative clones 2C10 (B6) and 3C5 ($\beta_2m^{-/-}$) were used. The histograms show CD8 expression and TCR expression in TCR^+ and $CD8^+$ cells, respectively.

GP33-specific $\beta_2m^{-/-}$ CTLs Have an Increased Avidity for H-$2D^b$ Molecules. Alloreactive CTLs from mice with low MHC class I expression have an increased avidity to self-MHC (23). By priming peptide-specific, self-MHC-restricted CTLs in $\beta_2m^{-/-}$ mice it is possible to study the role of self-MHC avidity for T cell specificity in MHC-restricted T cells. To compare the avidity for self-MHC class I in B6 and $\beta_2m^{-/-}$ CTLs specific for the GP33 peptide, these CTLs were tested for their ability to kill RMA target cells (MHC^{high}) in the absence of loaded peptides. B6 CTLs failed to kill RMA cells, whereas $\beta_2m^{-/-}$ CTLs efficiently killed these target cells (Fig. 3, A and B, respectively). Cold target competition experiments made with bulk CTLs showed that the $\beta_2m^{-/-}$ CTL killing of labeled RMA cells was completely inhibited by RMA-S pulsed with GP33 but not by RMA-S without peptide (Fig. 3 C). This showed that a majority of the clones in the $\beta_2m^{-/-}$ CTL population were specific for both self-MHC expressed at high levels and the peptide antigen presented by MHC at low levels.

Three GP33-specific $\beta_2m^{-/-}$ CTL clones were generated, all of which also killed RMA cells. However, the clone C10 was more efficient in killing RMA-S loaded with GP33 compared with RMA (Fig. 3 D), whereas clone 27/30 killed RMA slightly better than RMA-S plus GP33 (Fig. 3 E), and clone 3C5 killed both of these target cells to a similar extent (Fig. 3 F). This pattern is compatible with a clonal variation in TCR avidity for peptide versus MHC within the $\beta_2m^{-/-}$ CTL population. In line with the results obtained with bulk cultures, the GP33-specific B6 CTL clone 2C10 did not recognize RMA in the absence of GP33 peptide (Fig. 3 G).

To test the reactivity of the CTLs against MHC class I in the absence of peptides, we used RMA-S cells incubated at

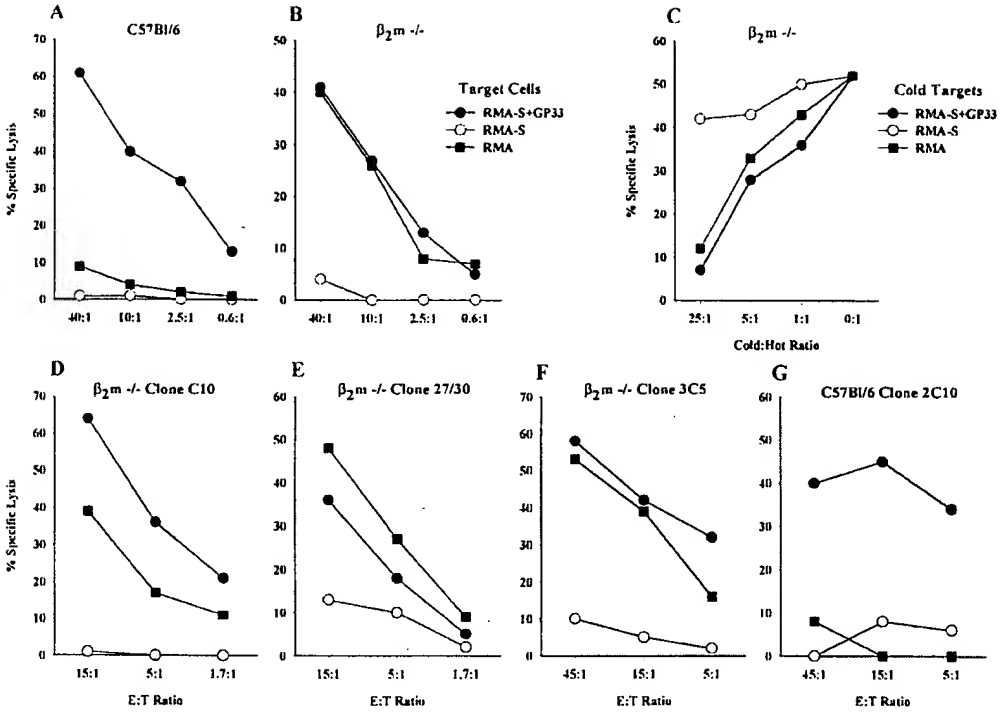

Figure 3. Elevated self-MHC reactivity in $\beta_2m^{-/-}$ CTLs. CTLs generated by synthetic LCMV GP33 peptide immunization (as described in Materials and Methods) in B6 (A) and $\beta_2m^{-/-}$ (B) mice were tested against RMA-S, RMA, and RMA-S loaded with GP33 in a ^{51}Cr-release CTL assay. (C) $\beta_2m^{-/-}$ GP33-specific CTL lysis of ^{51}Cr-labeled (hot) RMA target cells was subjected to cold target competition using cold (unlabeled) RMA-S, RMA, and RMA-S loaded with GP33. $\beta_2m^{-/-}$ CD8$^+$ CTL clones C10 (D), 27/30 (E), and 3C5 (F), and the B6 CTL clone 2C10 (G) were obtained by limiting dilution and tested against RMA-S, RMA, and RMA-S loaded with GP33.

cold temperature (26°C), which induce high levels of functionally "empty" MHC class I molecules (39), and T2D^b cells expressing D^b molecules mostly devoid of peptides. Clone 27/30 also killed RMA-S cells after cold temperature incubation (Fig. 4 A). Furthermore, T2D^b was recognized irrespective of loaded peptide, whereas T2 control targets were not killed (Fig. 4 B). Thus, the $\beta_2m^{-/-}$ CTL clone 27/30 displays an avidity for the restriction element of the GP33 peptide, even in the absence of specific peptide. It should be noted that these CTLs were primed to respond only against GP33, indicating that the ability to recognize self-MHC had been selected for during thymic development and priming. Although these results do not exclude that $\beta_2m^{-/-}$ CTLs also have an increased avidity for self-peptides, we use the term "peptide independent" to describe the specificity for the restriction element displayed by the GP33-specific $\beta_2m^{-/-}$ CTLs.

Triggering of GP33-specific $\beta_2m^{-/-}$ CTLs Uses a Higher Number of MHC Ligands to Compensate for Absence of Specific Peptide. We next compared the avidity of $\beta_2m^{-/-}$ CTLs for self-MHC with and without added GP33, by blocking of the H-2D^b ligands. We used an mAb specific for prop-

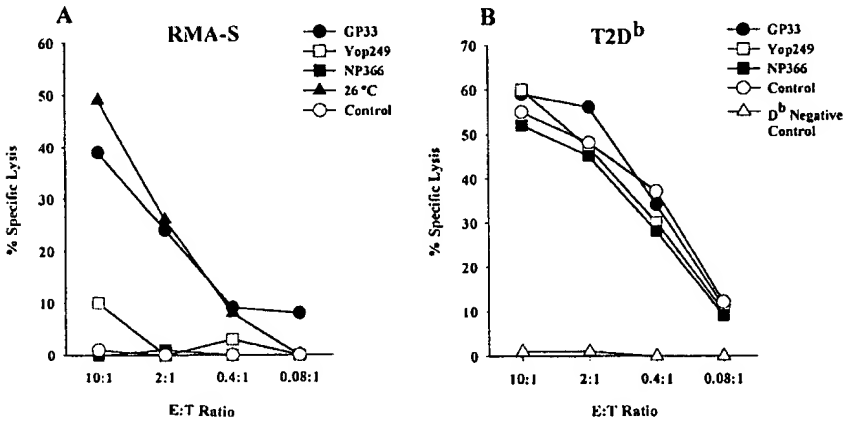

Figure 4. Recognition of self-MHC at high ligand density by the CTL clone 27/30 is largely independent of peptide. (A) The $\beta_2m^{-/-}$ CTL clone 27/30 was tested in a ^{51}Cr-release CTL assay against RMA-S target cells loaded with LCMV GP33 peptide, influenza NP366 peptide, Yop249 peptide, unloaded RMA-S, or RMA-S incubated for 12 h at 26°C without peptide to stabilize "empty" MHC class I on the cell surface. (B) Clone 27/30 was tested against T2D^b target cells loaded with GP33, NP366, Yop249, and unloaded T2D^b. The T2 cell line was included as a D^b negative control.

erly conformed H-2D^b molecules (B22-249.1), the binding of which is not affected by GP33 (data not shown). RMA target cells were killed at similar levels regardless of the presence or absence of specific peptide (Fig. 5). However, in the absence of GP33 peptide the CTL killing activity was efficiently blocked by 5.0 μg/ml of B22-249.1, whereas virtually no blocking was observed when the RMA target cells were prepulsed with the GP33 peptide at 37°C (Fig. 5 A). FACS® analysis of B22-249.1 binding to RMA cells did not allow accurate quantitation of the fraction of free H-2D^b ligands at half-maximal lysis, since half-maximal blocking of CTL lysis was achieved at a concentration at which staining was close to maximal (Fig. 5 B). However, these experiments clearly demonstrate that triggering of $\beta_2m^{-/-}$ CTLs in the absence of GP33 peptide requires a considerably higher number of H-2D^b ligands.

We next made a similar series of experiments using T2D^b cells as targets. These cells express the B22-249.1 epitope at levels <10% those of RMA (Table II) but are still killed by the $\beta_2m^{-/-}$ GP33-specific CTL clone 27/30 in the absence of specific peptide (Fig. 4 B). Lysis of T2D^b cells was blocked already at a 1.0 μg/ml concentration of B22-249.1 in the CTL assay (Fig. 5 C), indicating that a large fraction of cell surface H-2D^b molecules is required for triggering of this CTL clone. Pulsing of T2D^b cells with GP33 peptide prohibited blocking at all mAb concentrations tested (Fig. 5 C). This effect was peptide specific, since neither NP366 nor Yop249 had any effect on avidity as determined by blocking with mAb. By titration of B22-249.1 in FACS® analysis of T2D^b (Fig. 5 D), the fraction of free H-2D^b ligands on T2D^b at half-maximal peptide-independent lysis was estimated at ~80% (comparing Fig. 5, C and D). Thus, ~80% of the H-2D^b molecules on T2D^b were necessary for peptide-independent recognition by the high-avidity CTL clone 27/30, which corresponds to about four times the level of folded H-2D^b on $\beta_2m^{-/-}$ cells (Table II). Taken together, these data indicate that $\beta_2m^{-/-}$ CTLs use peptide-independent recognition of a high number of H-2D^b ligands to compensate for the absence of specific peptide.

GP33-specific $\beta_2m^{-/-}$ CTLs Are Less Dependent on the Exact GP33 Peptide Sequence. Finally, we analyzed the consequences of self-MHC avidity in GP33-specific B6 and $\beta_2m^{-/-}$ CTLs in terms of peptide specificity, when MHC was expressed at lower levels. Neither B6 nor $\beta_2m^{-/-}$ CTLs kill RMA-S (MHC^{low}) in the absence of specific peptide, and RMA-S cells require a pulse of ~100 pM GP33 peptide to become sensitive targets to both B6 and $\beta_2m^{-/-}$ CTLs (Fig. 6). This shows that B6 and $\beta_2m^{-/-}$ CTLs require roughly equal amounts of H-2D^b/GP33

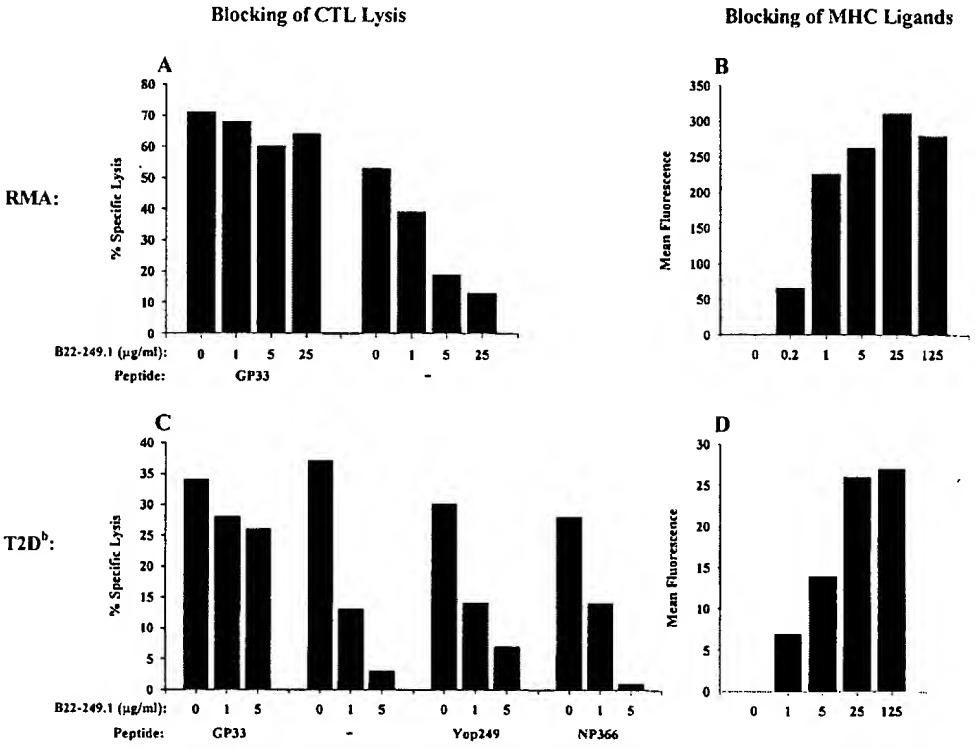

Figure 5. CTL triggering by self-MHC is a low-avidity event that requires interaction with a high density of ligands. (A) Recognition by $\beta_2m^{-/-}$ LCMV GP33-specific polyclonal CTLs of RMA in the absence or presence of GP33 peptide was blocked using the anti-H-2D^b conformation-specific mAb B22-249.1. (B) FACS® analysis of B22-249.1 titration on RMA cells. (C) Recognition by the GP33-specific $\beta_2m^{-/-}$ CTL clone 27/30 of T2D^b loaded with GP33, Yop249, NP366, or T2D^b without peptide was blocked using B22-249.1. (D) FACS® analysis of B22-249.1 titration on T2D^b. The preincubation of target cells with peptide at 37°C did not affect expression of H-2D^b in these experiments (data not shown).

Table II. *Surface Expression of H-2D^b on Cells with Defects in TAP or β_2m*

Cell type	H-2D^b expression (mean FL1)*	H-2D^b surface density (FL1/FSC)‡
B6§	227	398
TAP1$^{-/-}$	14	24
β_2m$^{-/-}$	4	6
TAP1/β_2m$^{-/-}$	0	0
RMA	396	568
RMA-S	15	21
T2D^b	28	31
T2	0	0

*H-2D^b expression was measured by FACS® analysis using the conformation-dependent B22-249.1 epitope.
‡As forward scatter (FSC) is proportional to the cross-sectional area of the analyzed cell, FL1/FSC was used as an estimate of the surface density of H-2D^b.
§B6, TAP1$^{-/-}$, β_2m$^{-/-}$, and TAP1/β_2m$^{-/-}$ Con A-activated blasts were generated by culturing splenocytes for 3 d in RPMI supplemented with 10% FCS and 5 μg/ml Con A.

complexes to get a triggering signal. However, when using GP33 peptide variants alanine-substituted at one or several TCR contact residues (reference 40, and data not shown), the β_2m$^{-/-}$ CTLs recognized RMA-S cells pulsed with up to 1,000-fold less peptide than B6 CTLs (Fig. 6). Substitution at one position (GP33-4A) already drastically reduced the efficiency of B6 CTLs against peptide-pulsed RMA-S targets, whereas β_2m$^{-/-}$ CTLs could still recognize peptides with alanine substituted at two positions. Thus, although β_2m$^{-/-}$ CTLs were still peptide specific, they were less dependent on the TCR contact residues in the peptide when the peptide was loaded on RMA-S target cells (which express about three times the levels of H-2D^b found on β_2m$^{-/-}$ cells [Table II]). These data indicate that the avidities for peptide and MHC both contribute functionally to the triggering of CTLs, and that they can be considered separately. Further, increased avidity for the restriction element compensates for a reduced avidity for the peptide.

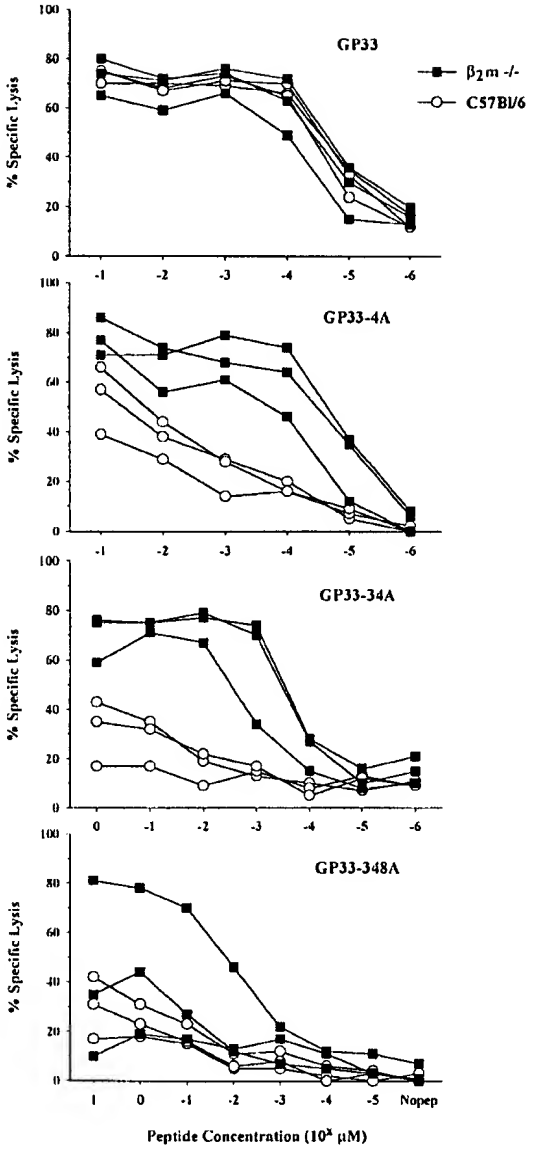

Figure 6. β_2m$^{-/-}$ CTLs display increased peptide promiscuity in recognition of MHC–peptide ligands. Groups of three B6 and three β_2m$^{-/-}$ mice were immunized with LCMV GP33 peptide as described in Materials and Methods. After 12 d, immune splenocytes were restimulated with GP33 for 6 d and tested in a ^{51}Cr-release assay against RMA-S target cells loaded with titrated amounts of GP33 and GP33 variants: GP33 KAVYNFATM; GP33-4A KAVANFATM; GP33-34A KAAANFATM; and GP33-348A KAAANFAAM.

Discussion

Contribution of Peptide-independent Recognition of MHC to the Specificity of T Cells. In this paper we have investigated the influence of TCR interaction with self-MHC in recognition of MHC-bound peptides. To address this issue, we used MHC class I–restricted CD8$^+$ T cells selected and primed in an environment with high (B6) or low (β_2m$^{-/-}$) MHC expression (25). We find that self-MHC can deliver a triggering signal independently of specific peptide, provided there is an increase in self-MHC density. The low surface density of H-2 expressed on RMA-S cells was not sufficient to trigger either β_2m$^{-/-}$ or B6 CTLs in the absence of the specific antigen, but increasing the ligand density by cold temperature incubation could sensitize RMA-S to the β_2m$^{-/-}$ CTL clone 27/30. Furthermore, since T2D^b cells were also killed irrespective of loaded peptide, recognition of syngeneic class I was most probably peptide independent. These results indicate that the interaction between TCR and self-MHC as observed in crystals not only provides the structural framework of specific T cell recognition, it also contributes a part of the avidity required for CTL triggering.

Recognition of MHC^{high} targets by $\beta_2m^{-/-}$ CTLs was blocked by mAb against properly conformed D^b molecules, whereas the presence of specific peptide prohibited blocking. These results indicated that the lack of GP33 was compensated by using an increased number of low-avidity interactions with self-MHC. The results further suggest that a minimum of four times the level of MHC class I expression present during selection and priming in vivo were necessary for activation by self-MHC to occur in vitro. RMA-S expresses only about three times the level of MHC found on $\beta_2m^{-/-}$ cells (Table II), which may explain why $\beta_2m^{-/-}$ CTLs were peptide specific when tested against RMA-S targets. In the presence of specific GP33 peptide, no mAb-mediated blocking of MHC^{high} target cell killing was observed. This supports the notion that MHC-restricted CTLs have a high avidity for the peptide antigen and a low avidity for self-MHC.

Interestingly, we found that T cells with an increased avidity for MHC class I molecules had a more relaxed peptide specificity, i.e., they were less dependent on the exact peptide sequence. While both B6 and $\beta_2m^{-/-}$ GP33-specific CTLs had similar sensitivity for GP33, $\beta_2m^{-/-}$ CTLs had superior sensitivity for peptides lacking one or several TCR contact residues of the GP33 peptide. Thus, increased recognition of the restriction element compensates for lack of TCR peptide affinity in recognition of low-affinity peptide ligands.

Our data argue that recognition of the two entities of the MHC complex, peptide and MHC heavy chain, can be considered separately (41). Increased recognition of MHC could substitute for lack of peptide recognition. The indication that TCR avidity for its ligand can be subdivided in this way opens the possibility of extending the differential avidity model of T cell selection and recognition (42).

Implications for the Avidity Threshold in T Cell Recognition. The present data can be interpreted within a model for T cell recognition based on the total avidity contributed by TCR affinity for MHC, affinity for peptide, and the number of MHC–peptide complexes. Increased avidity for MHC can compensate for lack of avidity for peptide, suggesting that these binding forces are functionally interchangeable and can be considered separately (Fig. 7 A). This would suggest a variation in the avidities for MHC and presented antigens among mature CTLs (See also Fig. 3, D–G). Interestingly, mice deficient in terminal deoxynucleotidyl transferase (TdT), which lack N-region additions in the TCR, display increased promiscuity in T cell recognition of peptide ligands (43). In this situation, MHC was suggested to provide compensatory avidity in recognition of peptide antigen by $TdT^{-/-}$ CTLs. Further, the data in this paper support an inverse correlation between the number of MHC–peptide ligands necessary for triggering and TCR avidity for the ligand (for data, see Fig. 5; for model, see Fig. 7 B). Lower avidity for an antigen would then be compensated by an increase in antigen density. This feature becomes most important for CTLs with a relatively high MHC avidity, since self-MHC can be regarded as a high-density and low-affinity ligand.

By combining Fig. 7, A and B, a hypothetical three-dimensional diagram can be generated in which the surface depicts how the TCR affinities for peptide and MHC, and the number of ligands necessary for triggering, are interchangeable (Fig. 7 C). The diagram proposes that when the TCR avidities for both peptide and MHC are low, the number of ligands necessary to achieve triggering will be high. We would like to suggest further that the same model would apply for thymic selection, where the threshold coordinates for positive selection would be considerably lower on all axes while coordinates for negative selection would be closer to the threshold for triggering of effector functions.

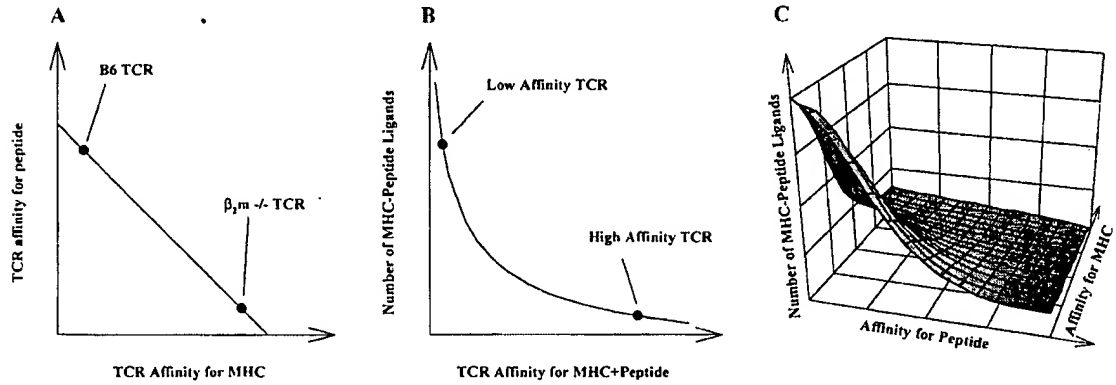

Figure 7. The TCR avidity threshold for T cell recognition. Hypothetical diagrams illustrating how the TCR affinities for peptide and MHC are interchangeable and mutually compensatory (A), and how the number of MHC–peptide ligands necessary for triggering inversely correlates with the TCR affinity for the ligand (B). (C) A and B are combined to generate a threshold composed of TCR affinities for MHC and peptide and the number of MHC–peptide complexes. In this threshold, all three contributing components can compensate for each other to generate the avidity necessary for T cell activation. The curve in A is presented as a straight line because a constant combined value of MHC–peptide avidity required for CTL triggering is subdivided into two parts, representing avidity for either MHC (x-axis) or peptide (y-axis). The shape of the curve in B is hypothetical. However, the hyperbolic shape will result if the total avidity required for triggering is constant, and if this avidity is a product of the number of ligands and the TCR affinity for each of these ligands (i.e., total triggering avidity = no. of ligands × TCR affinity for each ligand).

Aspects of CD8+ T Cell Selection in the Absence of β_2m. Selection of H-2D^b–restricted T cells specific for the GP33 epitope in $\beta_2m^{-/-}$ mice is thymus dependent, and results in a peripheral repertoire biased towards recognition of the restriction element expressed with and without self-peptides. However, peptide-independent triggering occurs only when MHC is expressed at levels considerably higher than those encountered by the T cells in vivo. This indicates repertoire calibration during thymic selection to fit the self-MHC ligand density in the periphery. Our results fit very well with the notion that T cells being selected in the thymus view self-MHC as a low-affinity ligand. Self-ligands capable of triggering induce deletion, and in the periphery self-MHC never reaches the ligand density to trigger CTLs in the absence of triggering antigens. It should be noted that in addition to the increased recognition of MHC, the $\beta_2m^{-/-}$ CTLs may also display an increased avidity for self-peptides. There may be a clonal variation in recognition of MHC and peptide within the $\beta_2m^{-/-}$ CTL population, where the clone 27/30 represents the most peptide-independent phenotype.

Considering the low expression of MHC present during selection in the $\beta_2m^{-/-}$ mice, it is possible that the CD8$^+$ T cells use upregulation of (co)receptor expression to achieve positive selection. Indeed, we have observed a marginal increase in CD8 expression in some of the $\beta_2m^{-/-}$ CTLs, which potentially could also contribute to the elevated recognition of self-MHC. However, in functional experiments based on recognition of GP33 and alanine-substituted variants, we have observed that $\beta_2m^{-/-}$ CTLs are less susceptible to CD8 blocking with anti-CD8α mAbs compared with B6 CTLs. This result would suggest, rather, a decreased dependency on CD8 in triggering of $\beta_2m^{-/-}$ CTLs (data not shown).

Accumulating evidence suggests that the CD8$^+$ T cells present in the $\beta_2m^{-/-}$ mice have retained important characteristics of the $\beta_2m^{+/+}$ wild-type concerning development and recognition of MHC. First, $\beta_2m^{-/-}$ mice can generate in vivo CTL responses against MHC class I–restricted peptide (44; and this study) and viral (45) antigens, and reject skin grafts over a minor histocompatibility barrier (46). Also, $\beta_2m^{-/-}$ CTLs can recognize peptide antigens on $\beta_2m^{+/+}$ targets (this study). Second, development of $\beta_2m^{-/-}$ CD8$^+$ T cells is dependent on the thymus (this study), and results in a CD8$^+$ T cell repertoire biased towards recognition of syngeneic class I (23; and this study). Third, H-2D^b heavy chains expressed in the absence of β_2m are recognized by conformation-dependent mAbs (27, 28, 47; and this study), and expression is TAP dependent (32; and this study). Note also that all detectable $\beta_2m^{-/-}$ CTL reactivity against self-MHC could be blocked by an mAb specific for properly conformed H-2D^b molecules, which reduces the likelihood for a role of aberrantly conformed free D^b heavy chains in this system. Fourth, wild-type CTLs can recognize endogenously processed antigens (48) and alloantigens (27, 48) on $\beta_2m^{-/-}$ cells. Taken together, these data suggest that T cell selection follows the normal rules in $\beta_2m^{-/-}$ mice, although the selection window is dramatically shifted towards low ligand density. The confrontation of such T cells with cells expressing normal MHC levels allows detection of the T cell avidity for self-MHC. However, the functional avidity must be established already during selection and must exist during priming. Thus, we propose that T cells in normal mice have a similar avidity for self-MHC which would be detectable by exposing them to cells with supraoptimal MHC levels.

We have demonstrated that avidity for self-MHC can trigger MHC-restricted T cells independently of specific peptide if ligand density of self-MHC is sufficiently increased. This interaction compensates for lack of TCR affinity for peptide, and it depends on a TCR–MHC interaction of relatively low affinity that requires high numbers of MHC ligands. The data and the avidity threshold model discussed contribute to our understanding of T cell specificity in the periphery and during thymic selection.

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Reactivity and Specificity of CD8$^+$ T Cells in Mice with Defects in the MHC Class I Antigen-Presenting Pathway

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INTRODUCTION

Major histocompatibility complex (MHC) class I molecules consist of a highly polymorphic membrane-spanning heavy chain of approximately 45 kD that is non-covalently associated with a light chain, β_2-microglobulin (β2m) (Bjorkman et al. 1987). The MHC class I molecules are expressed on the cell surface of almost all nucleated mammalian cells. They transport and present antigen in the form of short peptides, derived from intracellularly degraded proteins, to cytotoxic T cells (CTL) (reviewed in Yewdell & Bennink 1992). MHC class I presented peptides are usually 8-11 amino acids in length. A majority of them are generated in the cytosol by proteolytic degradation. Recent studies have suggested a role for a large enzyme complex termed the proteasome in this process (reviewed in Goldberg & Rock 1992). Two proteasomal subunits termed low molecular weight protein (LMP) 2 and 7 are encoded within the MHC gene complex. After proteolysis, peptide antigens are translocated into the lumen of the endoplasmic reticulum (ER) through the ATP-dependent transporter associated with antigen processing 1/2 (TAP1/2) (reviewed in Yewdell & Bennink 1992, Heemels & Ploegh, 1995).

Several strains of mutant mice with defects in the MHC class I antigen processing and presentation pathway have been generated by gene targeting technology over the last years. These include β2m -/- (Zijlstra et al. 1989, 1990, Koller & Smithies 1989, Koller et al. 1990), TAP1 -/- (Van Kaer et al. 1992), LMP2 -/-

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(Van Kaer et al. 1994) and LMP7 -/- mice (Fehling et al. 1994). These mice have not only been rewarding in the analysis of how these mutations affect MHC class I expression and MHC class I mediated antigen processing and presentation. They have also been valuable in studies of $CD8^+$ T cell development (this review; see also Raulet 1994) as well as NK cell development (Bix et al. 1991, Liao et al. 1991, Höglund et al. 1991, Ljunggren et al. 1994). This has been most evident in studies of β2m -/- and TAP1 -/- mice. Although low in numbers, $CD8^+$ T cells are clearly detectable in these mice. These $CD8^+$ T cells differ from similar cells in wild-type mice in that they have been selected on low levels of MHC class I molecules. As a consequence, they possess specificities not shared by $CD8^+$ T cells in corresponding wild-type mice. This results in a $CD8^+$ T cell population with a bias towards reactivity with "self" MHC class I expressed at a normal ligand density. The $CD8^+$ T cells in the TAP1 -/- and β2m -/- mice may thus serve as a tool to analyze the "self" MHC bias of the T cell repertoire, a somewhat unexpected development for the first "MHC class I deficient" mice.

The main purpose of the present review is to discuss the development of $CD8^+$ T cells in mice with defects in the MHC class I pathway of antigen processing and presentation, in particular with respect to the reactivity and specificity of $CD8^+$ T cells selected in this environment. A particular emphasis will be devoted to studies with β2m -/- mice, and results generated in this model will be compared with results generated in the TAP1 -/- and TAP1/β2m -/- mouse models. Results generated in studies of LMP2 -/- and LMP7 -/- mice will also be discussed briefly.

T CELL SELECTION

T cell precursors are derived from the bone marrow and mature in the thymus. During this maturation T cells go through a complex, and as yet not completely understood, selection process where "useless" and "harmful" T cells are deleted while "useful" T cells are spared and released to a life in the periphery (Von Boehmer et al. 1989). Two types of cellular selection in the thymus are central to the repertoire determination of T cells (Janeway 1994). First, T cells acquire the capacity to recognize antigens in the context of MHC molecules through a process referred to as positive selection (reviewed in von Boehmer 1994, and Jameson et al. 1995). This process is dependent on interactions of the TCR with MHC molecules expressed by the selecting cells of the thymus. Engagement of the TCR with MHC class I glycoproteins is required for immature T cells to differentiate into $CD4^-8^+$ cytotoxic T cells, whereas TCR engagement with class II molecules is required for differentiation to the $CD4^+8^-$ helper T cell lineage. Second, potentially autoreactive T cells are eliminated from the mature T cell repertoire through negative selection (reviewed in Nossal 1994). In addition to T cell selection in the thymus, a substantial number of T cells may also be selected extrathymically, for example in the gut (reveiwed in Rocha et al. 1995).

MHC CLASS I EXPRESSION IN THE ABSENCE OF β2M AND TAP GENE PRODUCTS

Both β2m and peptides provided by the TAP transporter are necessary for normal cell surface expression of MHC class I molecules (reviewed in Bijlmakers & Ploegh 1993). This first became evident through studies of mutant cell lines and was then more recently confirmed with cells derived from β2m -/- as well as TAP1 -/- mice. However, neither a TAP deficiency nor a β2m deficit is sufficient to totally abolish expression of H-2K^b or D^b class I molecules on the surface of cells of the H-2^b haplotype. Cells devoid of β2m express a limited number of free H-2D^b (Allen et al. 1986, Zijlstra et al. 1990, Vitiello et al. 1990, Bix & Raulet 1992, Glas et al. 1992) as well as H-2K^b class I heavy chains (Glas et al. 1992, Hogquist et al. 1993, Machold et al. 1995), at least some of which appear to be occupied by peptide (Glas et al. 1992, Machold et al. 1995). Similar observations have been made with other class I alleles, such as H-2L^d (see e.g. Cook et al. 1995). Cells devoid of TAP gene products express reduced levels of class I heavy chains complexed with β2m, either devoid of peptide, or occupied by peptides that bind to class I molecules independently of the TAP complex (Ljunggren et al. 1990). Both TAP and β2m deficient cells are recognized by allospecific CTL (see e.g. Öhlén et al. 1990, Bix & Raulet 1992, Glas et al. 1992). In addition, β2m deficient cells can be efficiently recognized by MHC class I restricted cells (Vitiello et al. 1990, Glas et al. 1992, Hogquist et al. 1993, Lehmann-Grube et al. 1994, Zügel et al. 1994, Cook et al. 1995). Taken together, these observations clearly indicate that cells with mutations in the β2m or TAP genes still do express MHC class I molecules, though at levels that are significantly lower than on wild-type cells. This notion is of significant importance in the following discussion since it implies that residual levels of MHC class I molecules expressed in β2m -/- and TAP1 -/- mice may function as ligands on cells involved in CD8⁺ T cell selection.

CD8⁺ T CELL DEVELOPMENT IN β2M -/- MICE - INITIAL OBSERVATIONS

The β2m -/- mice were generated simultaneously by two independent laboratories (Zijlstra et al. 1989, 1990, Koller & Smithies, 1989, Koller et al. 1990). One of their most remarkable features was their MHC class I deficient phenotype *per se*, yet their ability to reproduce, develop without significant physiological alterations, and survive in a non-germ free environment. Initial characterization of the β2m -/- mice revealed no gross abnormalities in the development of TCR γ/δ⁺, CD4⁺CD8⁺ and CD4⁻CD8⁻ T cells. However, the initial reports indicated a total lack of TCR α/β⁺ CD4⁻CD8⁺ T cells in the thymus and lymphoid organs and no detectable CD8⁺ T cell mediated cytotoxicity (Zijlstra et al. 1990, Koller et al. 1990). Subsequent studies demonstrated significant effects imposed by the β2m mutation on other T cells subsets as well, including TCR α/β⁺ CD4⁻CD8⁻ T cells in the thymus and TCR α/β⁺ CD8⁺ T cells in the intestinal epithelium (Correa et al. 1992, Bix &

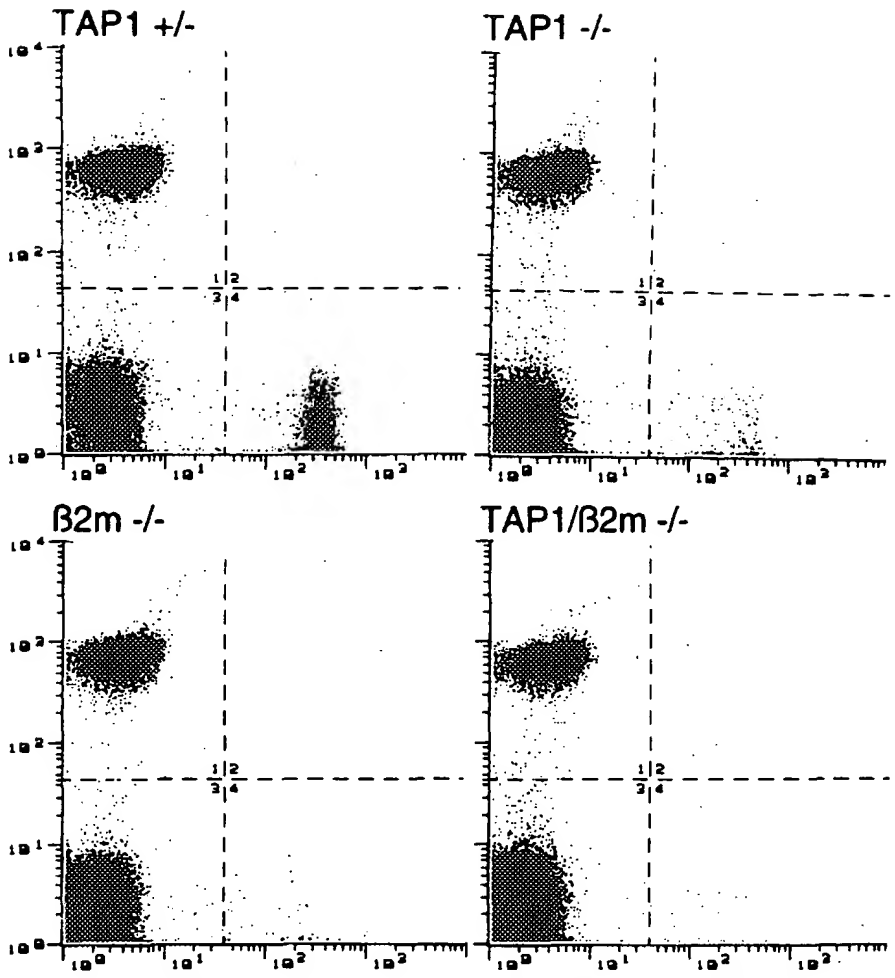

Figure 1. CD4 and CD8 expression on peripheral blood leukocytes from wild-type as well as TAP1 -/-, β2m -/-, and TAP1/β2m -/- mice (X=CD8, Y=CD4). All mutant mice have $CD8^+$ T cells, although numbers are significantly lower than in wild-type mice. Mean percentage of positive cells in peripheral blood was: wild-type 11.2 (S.D. 1.00), TAP1 -/- 0.83 (S.D. 0.24), β2m -/- 0.20 (0.07) and TAP1/β2m -/- 0.15 (0.05) (n=10, for TAP1 -/- mice n=20). Data from Ljunggren et al. 1995a.

Raulet 1993, Neuhaus et al. 1995; reviewed in Raulet 1994). In contrast, most TCR γ/δ^+ T cells appeared to develop normally in β2m -/- mice (Zijlstra et al. 1990, Koller et al. 1990, Correa et al. 1992), even though some TCR γ/δ^+ T cell subsets were affected by the β2m mutation (Wells et al. 1991, Pereira et al. 1992).

β2M -/- MICE ARE NOT DEVOID OF CD8⁺ T CELLS

The remarkable reduction in the numbers of mature CD8⁺ T cells in the thymus and in the peripheral lymphoid organs of the β2m -/- mice confirmed the notion that interactions with MHC class I molecules are essential for normal development of these cells (reviewed in von Boehmer 1994, and Jameson et al. 1995). As a consequence of these early reports, the β2m -/- mice were rapidly established as a model for assessment of various immune responses in the absence of CD8⁺ T cells (reviewed in Raulet 1994). However, within a couple of years, the initially reported CD8⁺ T cell deficient state of the β2m deficient mice was challenged. In a number of studies, it was observed that CD8⁺ T cells in β2m -/- mice mounted vigorous MHC class I specific responses against experimental tumors or allogeneic splenocytes *in vivo* (see e.g. Apasov & Sitkovsky 1993, 1994, Lamousé-Smith et al. 1993, Glas et al. 1994, Udaka et al. 1994). Refined analysis by flow cytometry also revealed the existence of a small pool of CD8⁺ T cells in naive β2m -/- mice (illustrated in Fig. 1).

In the first published reports with experimental tumor grafts, peritoneal exudate cells from β2m -/- mice which had been injected intraperitoneally with allogeneic tumor cells were shown to contain a large proportion of mature CD8⁺ T cells with MHC class I specific cytotoxic activity (Apasov & Sitkovsky 1993). This report was followed by two other reports demonstrating that β2m -/- mice were fully capable of rejecting high dose challenges of allogeneic tumors, while *in vivo* depletion of either CD4⁺ or CD8⁺ T cells (or both) resulted in susceptibility to tumor growth (Lamousé-Smith et al. 1993, Apasov & Sitkovsky 1994). *In vitro* experiments in these studies demonstrated the presence of cytotoxic MHC class I specific CD8⁺ T cells. Similar results from our and other laboratories independently confirmed these observations (Glas et al. 1994, Udaka et al. 1994). Taken together, these studies indicated that the β2m -/- mice had low but clearly detectable numbers of CD8⁺ T cells, and that this pool of CD8⁺ T cells had the capacity to rapidly expand and react upon antigenic stimulation.

THE CD8⁺ T CELL REPERTOIRE IN β2M -/- MICE IS BIASED TOWARDS REACTIVITY WITH SELF MHC CLASS I

The origin of the CD8⁺ T cells detected in the β2m -/- mice was not known. It was speculated that they could develop either through positive selection on low levels of MHC class I molecules in the thymus or, alternatively, represent a small population of CD8⁺ T cells that differentiated independently of class I recognition (Apasov & Sidkovsky 1993, 1994, Lamousé-Smith et al. 1993, reviewed in Raulet 1994). In a more detailed analysis of this phenomenon, we observed that the CD8⁺ T cells generated in β2m -/- mice against allogeneic tumor or lymphoid cells always cross reacted and killed cells expressing "self" MHC class I when expressed at normal levels, i.e. when associated with β2m (Glas et al. 1994; illustrated in Ta-

TABLE I

CD8⁺ T cell responses in β2m -/- mice - CD8⁺ T cells in β2m -/- mice have a bias towards reactivity with "self" class I expressed at a normal ligand density

		Target cells		
Effector cells[1]	E:T ratio	P815 (H-2^d)	RMA (H-2^b)	RMA-S (H-2^b low)
β2m -/-	10:1	52 %[2]	77 %	20 %
anti-BALB/c	2:1	33 %	44 %	9 %
	0.4:1	16 %	20 %	5 %
	0.08:1	6 %	12 %	1 %
β2m -/-	10:1	11 %	73 %	27 %
anti-C57BL/6	2:1	14 %	58 %	15 %
	0.4:1	9 %	33 %	9 %
	0.08:1	0 %	15 %	2 %

[1] β2m -/- mice of the H-2^b haplotype. Effectors generated by *in vivo* priming and *in vitro* restimulation of immune splenocytes as described in Glas et al. 1994. [2]Percent specific

ble I). Yet, these CD8⁺ T cells were always specific for the allogeneic class I molecules that were used to prime the mice with (whether being of the H-$2^{d,k,a\ or\ s}$ haplotypes). This bias towards reactivity against syngeneic MHC class I expressed at a normal ligand density strongly indicated that at least a part of the residual CD8⁺ T cell population had been selected on the low levels of class I molecules expressed in the β2m -/- mice (see separate discussion in the section on models and interpretations).

The bias towards reactivity with syngeneic H-2^b class I molecules expressed at a normal ligand density became particularly clear in studies with TAP-deficient RMA-S cells which normally express low levels of class I molecules. In most experiments, RMA-S cells showed a low, somewhat variable sensitivity against allospecific CTL generated in β2m -/- mice (Table I). However, when RMA-S cells were incubated with exogenous peptides (of viral or other defined origin) or exposed to reduced temperature, both of which results in increased levels of H-2^b class I molecules at the cell surface (Townsend et al. 1989, Ljunggren et al. 1990), they became sensitive to similar anti-H-2^d allospecific CTL. Cold target competition experiments confirmed that many of the allospecific CD8⁺ T cells in the β2m -/- mice had a dual specificity in that they killed the allogeneic targets as well as target cells expressing syngeneic class I when associated with β2m, and thus having class I molecules expressed at the cell surface at normal levels (Glas et al. 1994).

Anti-H-2^b reactive CTL could also be elicited in the β2m -/- mice by direct priming with H-2^b class I positive cells (Glas et al. 1994), an observation that was also made by Walden and colleagues (Udaka et al. 1994). In our studies of the CD8⁺ T cells in the β2m -/- mice, we observed that the TCR α/β⁺ CD8⁺ responder T cells readily killed H-2^b class I positive targets while they spared class I deficient β2m -/

TABLE II

Summary of CD8⁺ T cell responses in β2m -/- mice

	Stimulator cells		Target cells	
Mice[1]	*In vivo* priming	*In vitro* restimulation	Allogeneic	Syngeneic
β2m +/-		allogeneic	+[2]	–
"	allogeneic	allogeneic	+	–
"		syngeneic	–	–
"	syngeneic	syngeneic	–	–
β2m -/-		allogeneic	–[3]	–
"	allogeneic	allogeneic	+	+
"		syngeneic	–	+
"	syngeneic	syngeneic	–	+

[1]Effector cells from β2m +/- and β2m -/- mice. Data summarized from Glas et al. 1994 as well as unpublished results. [2] +, lysis; -, no lysis. [3]No or only weak cytotoxic responses.

- targets and third party targets. They were thus specific for "self" MHC class I, but only when expressed at a normal ligand density. Studies with H-2K^b or D^b transfected human cells lines verified the MHC class I specificity in the response. CTL reactivity against TAP deficient RMA-S, T2K^b and T2D^b cells further suggested that the CTL reactivity may be largely independent of TAP-dependent peptides, arguing against the idea that the response elicited in the β2m -/- mice was solely directed against peptide antigens not shared by the β2m -/- mice (see below).

Subsequent studies demonstrated that CD8⁺ T cells from β2m -/- mice could be induced in a primary mixed lymphocyte culture (MLC) after stimulation with H-2^b class I positive cells, whereas allogeneic cells generally failed to elicit a response under similar conditions (illustrated in Table II). In the latter case, *in vivo* priming was necessary for the generation of efficient responses. In retrospect, this observation may explain why Zijlstra et al. as well as Koller et al. in their initial studies failed to detect CD8⁺ T cell mediated reactivity in the β2m -/- mice (Zijlstra et al. 1990, Koller et al. 1990). The anti-H-2^b reactivity elicited in β2m -/- mice of the H-2^b haplotype was abolished when β2m -/- T cells matured in an environment with normal MHC class I expression, as assessed in bone marrow chimeric mice (Glas et al. 1994). More recent results have also demonstrated that the generation of alloreactive, and anti-H-2^b class I reactive CD8⁺ T cells in the β2m -/- mice is largely thymus dependent (Glas et al., unpublished data), indicating a clear role for the thymus in the selection of functional CD8⁺ T cells in the absence of β2m. Taken together, these results suggested that events occurring during development influence the self reactivity of β2m -/- CD8⁺ T cells. The above-mentioned results are not only observed in mice of the H-2^b haplotype. β2m -/- mice of the H-2^d and H-2^k haplotypes also have CD8⁺ T cells with increased reactivity towards syngeneic class I H-2^d and H-2^k molecules, respectively (Cook et al. 1995, Glas et al., unpublished data). Interestingly, CTL from β2m -/- mice of the H-2^d haplotype reacted

most strongly with H-2L^d, less against H-2K^d and not at all against H-2D^d (Glas et al., unpublished data).

Taken together, these results have been taken as indirect evidence of a novel specificity of the CD8$^+$ T cells in β2m -/- mice: an ability to specifically recognize and kill target cells expressing syngeneic MHC class I expressed a normal ligand density. It was speculated that expression of low levels of MHC class I molecules on selecting cells led to positive selection of a small pool of CD8$^+$ T cells with very high affinity for "self" class I. Due to low levels of MHC class I molecules, it was further speculated that few (if any) of these cells meet the requirements for negative selection, resulting in a CD8$^+$ T cell pool prone to be "autoreactive" in a β2m positive littermate (Glas et al. 1994). However, it cannot be excluded that some of these results were consequences of reactivities against class I presented peptides, other than those that were presented on the MHC class I heavy chains in the β2m -/- mice. These, not mutually exclusive, models are discussed below in the section on "models and interpretations".

SPLIT TOLERANCE OF CD8$^+$ T CELLS FROM β2M -/- MICE?

Before the above mentioned results were published, Zijlstra and colleagues as well as Markmann and colleagues reported that β2m -/- mice had the capacity to reject syngeneic MHC class I positive skin grafts. At that time, however, these results were difficult to explain since the β2m -/- mouse model still was considered to be a model in which no CD8$^+$ T cells did develop (Zijlstra et al. 1992, Markmann et al. 1992). The discovery of a residual CD8$^+$ T cell repertoire in the β2m -/- mice, and the strong bias of these CD8$^+$ T cells towards reactivity with syngeneic class I molecules expressed at a high ligand density, strongly suggests that CD8$^+$ T cells could contribute to the rejection response against syngeneic MHC class I positive skin grafts in the β2m -/- mice. This interpretation was also used to explain the rejection of MHC class I positive skin grafts in TAP1/β2m -/- (double mutant) mice (Ljunggren et al. 1995a).

However, the idea of a "self" biased CD8$^+$ T cell repertoire in β2m -/- mice is not fully compatible with the inability of β2m -/- mice to reject certain H-2^b expressing tumor grafts, an observation that was initially made by Ostrand-Rosenberg and colleagues (Lamousé-Smith et al. 1993), and later confirmed by ourselves not only in β2m -/- but also in TAP1 -/- and TAP1/β2m -/- mice (Freland, Chambers and Ljunggren; unpublished results). In a more detailed analysis of this phenomenon, Vukmanovic and colleagues analyzed the ability of CD8$^+$ T cells from β2m -/- mice to proliferate in response to cells expressing self MHC class I expressed at a normal ligand density (Jhaver et al. 1995). While β2m -/- CD8$^+$ T cells killed target cells expressing syngeneic class I expression at a normal ligand density, the T cells did not proliferate and did not secrete cytokines (e.g. IFN-γ or IL-3/GM-CSF) when stimulated with these targets. The inability to generate CD8$^+$ T cell lines from β2m -/- mice by repeated stimulation with syngeneic MHC class I

positive cells, such as the EL-4 lymphoma line, supported this notion (Jhaver et al. 1995). In contrast, stimulation of $CD8^+$ T cells from β2m -/- with allogeneic cells induced vigorous growth of CTL lines. Furthermore, $H\text{-}2^h$ class I expressing tumor cell lines that were accepted in β2m -/- mice of the $H\text{-}2^h$ haplotype were readily rejected by $CD8^+$ T cells in β2m -/- mice of the $H\text{-}2^d$ and $H\text{-}2^k$ haplotypes (Vukmanovic et al., personal communication; Freland, Chambers and Ljunggren, unpublished results). These results suggest the existence of a state of partial but not complete tolerance of the $CD8^+$ T cells in β2m -/- mice, leading to acceptance of $H\text{-}2^h$ tumors despite strong cytotoxic activity. To explain the discrepancy in the ability to reject skin grafts but not tumor grafts, it was speculated that generation of cytotoxic responses might be sufficient to reject skin grafts despite no (or only moderate) proliferate responses, whereas eradication of rapidly growing tumors may require additional mechanisms such as the ability to rapidly proliferate and secret cytokines (Jhaver et al. 1995).

The generality of this partial tolerance remains to be investigated. For example, in a model based on β2m -/- mice of the $H\text{-}2^d$ haplotype, Hansen and colleagues demonstrated that $CD8^+$ T cells readily proliferated in response to a tumor expressing syngeneic class I expressed a normal ligand density. It was suggested that these T cells either responded specifically against peptides expressed by the tumor cell line, or against the class I molecules expressed at wild-type levels in a peptide-independent manner (Cook et al. 1995). More recently, this group has generated several β2m -/- anti-wild-type $CD8^+$ T cell lines (J.R. Cook, personal communication). Thus, the issue of a partial state of self tolerance in the $CD8^+$ T cell repertoire in β2m -/- mice may, at least partially, depend on the experimental model employed (see also the section on TAP1 -/- mice). It is tempting to speculate that a (strong) peptide mismatch may break the partial state of tolerance. In such a scenario, the proliferative responses would be limited towards non-antigenic tumors and be more pronounced towards antigenic tumors. Other factors, such as the influence of a concomitant class II response (L.D. Fast, personal communication), may also explain the differences in results observed in different laboratories and in different experimental models.

MHC CLASS I RESTRICTED RESPONSES IN β2M -/- MICE

From the discussion above, it is clear that the β2m -/- mice possess a small pool of $CD8^+$ T cells. Although these display an unusual repertoire in that they react with cells expressing normal levels of "self" MHC class I, they clearly also respond to allogeneic MHC class I even when expressed at a low ligand density (Glas et al. 1994). This appears adequate since it can be argued that these T cells have been selected to operate in an environment where all cells express low levels of MHC class I molecules. An important question is if these $CD8^+$ T cells are functional during a virus infection or against tumors in the same host, i.e. when antigens are presented by β2m -/- MHC class I molecules in a restricted fashion. There are only

a few studies in which this issue has been addressed directly. Most of the initial studies of virus infections in β2m -/- mice were interpreted under the assumption that these mice were devoid of $CD8^+$ T cells, thus focusing on the role of $CD4^+$ T cells in the control of disease development (reviewed in Doherty 1993). In the course of these studies, it was observed that β2m -/- mice readily cleared many virus infections. These include vaccinia virus (Spriggs et al. 1992), the generally non-lethal HKx31 influenza A virus (Eichelberger et al. 1991) as well as low levels of the virulent PR8 influenza A virus and Sendai virus (Bender et al. 1992, Hou et al. 1992). Many of these effects have been linked to the action of $CD4^+$ T cells. In contrast to the above-mentioned viruses, the β2m -/- mice show a significantly reduced capacity to clear the systemic infections caused by Theilers virus (Fiette et al. 1993) and lymphocytic choriomeningitis virus (LCMV). In the case of LCMV infection, the acute fatal immunopathology seen in classical choriomeningitis virus infections is changed to a chronic wasting disease with low mortality (Muller et al. 1992, Lehmann-Grube et al. 1993, Doherty et al. 1993, Quinn et al. 1993).

To our knowledge, there has been no clear cut demonstration of clearance of viral infections mediated by $CD8^+$ T cells in β2m -/- mice. More recent studies on the role of $CD8^+$ T cells in the immunopathology of Sendai virus infections in β2m -/- mice have underscored the notion that $CD8^+$ T cells are largely unable to eliminate virus infected lung epithelial cells (Hou & Doherty, 1995). However, despite this observation, respectable levels of MHC class I restricted, Sendai virus specific, cytotoxicity were observed in these mice (Hou et al. 1992; Doherty, personal communication). Yet, despite the detection of MHC class I restricted virus specific T cells, the overall conclusions from these and similar studies must be that $CD8^+$ T cells generated in the β2m -/- mice cannot in any significant way deal with at least Sendai virus or LCMV infections in the β2m -/- environment (Doherty, personal communication).

It has been reported that infusion of immune spleen cells from syngeneic β2m +/+ mice results in clearance of lymphocytic choriomeningitis virus (LCMV) in β2m -/- mice (Lehmann-Grube et al. 1994), although approximately five times more cells were needed to achieve antiviral effects in β2m -/- mice than in wild type mice. Depletion of the immune splenocytes of $CD8^+$ T cells abolished the anti-viral potential. Similar observations were made in studies of the chronic wasting disease that LCMV induces in β2m -/- mice (Doherty et al. 1993). When immune $CD8^+$ T cells were transferred to $CD4^+$ T cell depleted β2m -/- mice, it was demonstrated that these $CD8^+$ T cells could induce significant levels of meningitis in virus infected β2m -/- recipients. These results were taken as evidence for the ability of LCMV infected β2m -/- cells to present viral antigen *per se* to $CD8^+$ T lymphocytes *in vivo*, and for the ability of the latter to (at least partially) clear viral infection in an environment lacking β2m.

While it has been relatively difficult to detect MHC class I restricted $CD8^+$ T cell responses after virus infections in β2m -/- mice, peptide immunizations in β2m -/- mice have been more successful. Peptide-specific CTL have been elicited in β2m

deficient mice of both H-2^d (Cook et al., 1995) and H-2^b backgrounds (Sandberg et al., unpublished data). The responses were highly specific for the given peptide, despite the significant reduction in MHC class I expression in the mice. At least some peptide-specific CTL lines generated from β2m -/- mice of the H-2^d haplotype showed altered affinities for their peptide ligands, when compared to similar CTL lines generated in corresponding wild-type mice (Cook et al. 1995).

At last, one example of what might be an MHC class I restricted T cell response *in vivo* in β2m -/- mice was the demonstration of skin graft rejection responses over multiple minor histocompatibility differences in an environment totally devoid of β2m. More specifically, β2m -/- mice of 129/Sv and C57BL/6 backgrounds (both of an H-2^b background), respectively, rejected each other's skin grafts (Zijlstra et al. 1992). However, at this time the existence of a $CD8^+$ T cell pool capable of mediating rejection responses was not appreciated, and the phenomenon was not explored in detail. Skin graft responses, as well as other responses against solid organ grafts, are often complex in nature and far from understood in detail (reviewed in Auchincloss et al. 1989). Yet, with available knowledge in hand, it is tempting to speculate that MHC class I restricted $CD8^+$ T cells might have contributed to the rejection responses observed.

$CD8^+$ T CELL DEVELOPMENT IN TAP1 -/- MICE

Some two and a half years after the generation of β2m -/- mice, mice devoid of TAP1 were described (Van Kaer et al. 1992). Cells from TAP1 -/- mice are defective in intracellular assembly and cell surface MHC class I expression. These properties were strikingly similar to those observed in the first studies of TAP2 deficient RMA-S cells (Ljunggren & Kärre 1985, Kärre et al. 1986, Ljunggren et al. 1989). In further analysis of RMA-S cells (Townsend et al 1989, Ljunggren et al. 1990), it was observed that cell surface MHC class I expression could be induced by treatment of the cells with presentable peptide or by culture at reduced temperature. Similar observations were made with cells from TAP1 -/- mice (Van Kaer et al. 1992). Furthermore, as observed for RMA-S cells (Townsend et al. 1989, Öhlén et al. 1990), TAP1-deficient cells were unable to present intracellular protein antigens to class I restricted cytotoxic T cells (Van Kaer et al. 1992). As initially reported also for β2m -/- mice, they were described to have a normal distribution of $CD4^+8^-$ and $CD4^+8^+$ T cells and to be severely deficient in $CD4^-8^+$ T cells (Van Kaer et al. 1992). Initial analysis of $CD8^+$ T cell activity also suggested that spleen cells from these mice were unable to induce CTL responses against alloantigens (Van Kaer et al. 1992). This defect was later found to be relative rather than absolute (Aldrich et al. 1994, Ljunggren et al., 1995a and 1995b, discussed in Ljunggren & Van Kaer 1995).

As observed in the β2m -/- mice, residual $CD8^+$ T cells in TAP1 -/- mice (but not TAP1 +/- littermates) were able to generate strong cytotoxic responses against cells expressing syngeneic H-$2K^b$ and D^b class I molecules (Aldrich et al. 1994). In line

with the observations in β2m -/- mice, these responses could be further potentiated by *in vivo* priming. TAP1 -/- mice primed and restimulated with allogeneic H-2^d expressing splenocytes generated anti-H-2^d specific responses which were nearly as strong as those observed in TAP1 +/- (class I positive) littermates. TAP1 -/- anti-H-2^b and anti-H-2^d T cells did not recognize TAP1 -/- lymphoblasts or TAP2 deficient RMA-S target cells, unless class I expression was induced by treatment at low temperature (Aldrich et al. 1994). Notwithstanding the profound effect observed by the TAP1 mutation on selection of $CD8^+$ T cells (Van Kaer et al. 1992), these observations suggested that the TAP1 -/- mice were able to select $CD8^+$ anti-H-2^b and alloreactive T cells in the absence of a functional TAP complex (Aldrich et al. 1994). As recently observed in β2m -/- mice, TAP1 mice were also able to generate peptide specific CTL responses (Sandberg et al. 1996). These results will be discussed below in the context of class I bound peptides and positive selection.

Anti-H-2^b reactive T cells from β2m -/- mice (of the H-2^b haplotype) were, as discussed above, reported to be largely unable to proliferate and produce cytokines upon stimulation with syngeneic H-2^b expressing cells (Jhaver et al. 1995). This observation differs quite significantly from the observations made with TAP1 -/- mice (Aldrich et al. 1994). After *in vivo* priming with H-2^b splenocytes, the numbers of $CD8^+$ T cells increased slightly. However, after *in vivo* priming and *in vitro* restimulation with H-2^b expressing cells, the numbers of $CD8^+$ T cells increased dramatically. More than 50% of the cells in the mixed lymphocyte culture were $CD8^+$ T cells, indicating (but not formally proving) that these cells had proliferated upon stimulation. Future studies may reveal whether differences in experimental conditions, or the different mutations in the antigen presentation pathway *per se*, account for these differences.

COMPARISON OF $CD8^+$ T CELL DEVELOPMENT AND REACTIVITY IN β2M -/- AND TAP1 -/- MICE

Although TAP1 -/- and β2m -/- mice in many respects appear similar (Zijlstra et al. 1990, Koller et al. 1990, Van Kaer et al. 1992, Glas et al. 1994, Aldrich et al. 1994), the expression of class I molecules, and thus also the T cell selection ligands, differ quantitatively and qualitatively between the two types of mice. To address whether this affects the outcome of the T cell selection process, we made a side by side comparison of the numbers and reactivities of $CD8^+$ T cells in these mice (Ljunggren et al. 1995b). TAP1 -/- mice had relatively higher numbers of $CD8^+$ T cells than β2m -/- mice. The responses of residual $CD8^+$ T cells in TAP1 -/- and β2m -/- mice were also different: (i) Alloreactive CTL responses from TAP1 -/- mice were generally stronger than those from β2m -/- mice. (ii) Alloreactive CTL from β2m -/- mice killed cells expressing normal H-2^b at levels which were at least equal, and in most experiments even stronger, than the cross-reactivity of allospecific CTL from TAP1 -/- mice. (iii) β2m -/- mice always responded more strongly than TAP1 -/- mice when immunized with, and tested against, H-2^b expressing targets. (iv)

β2m -/- anti-H-2^b (B6) reactive CD8⁺ CTL reacted with TAP-deficient targets, whereas TAP1 -/- anti-H-2^b (B6) CTL, as expected, were tolerant against the same targets. (v) β2m -/- mice were also able to respond directly to TAP1 -/- cells, while no reactivity was observed in the opposite direction (Ljunggren et al. 1995b). Taken together, the differences in numbers of CD8⁺ T cells, their ability to react with alloantigens and their cross-reactivity with normal H-2^b class I led us to speculate that they were caused by differences in expression of MHC class I ligands on selecting cells in the thymus (further discussed below; Ljunggren et al. 1995b). Yet, having highlighted these difference between the TAP1 -/- and β2m -/- mice, it is important to stress that the features they share with respect to selection of CD8⁺ T cells are more prominent than the differences.

TAP1 -/- MICE SELECT A CD8⁺ T CELL REPERTOIRE THAT IS BOTH DIVERSE AND PEPTIDE SPECIFIC

Given the fact that TAP1 -/- mice can select a functional CD8⁺ T cell repertoire, and the notion that these T cells must have been selected on class I ligands that are largely devoid of TAP dependent peptides, we recently set out to investigate the diversity and specificity of the peripheral CD8⁺ T cell population in these mice (Sandberg et al. 1996). Although low in numbers, CD8⁺ T cells in TAP1 -/- mice were polyclonal with regard to TCR Vβ chain expression. A detailed analysis revealed that Vβ usage in TAP1 -/- mice was strikingly similar to that in wild-type mice, with only relatively minor exceptions. As observed in studies of β2m -/- mice (Cook et al. 1995, Sandberg et al. unpublished), the CD8⁺ T cells in TAP1 -/- mice readily mounted epitope specific CTL responses against several well defined MHC class I restricted peptides. To date, seven out of eight H-$2K^b$ or D^b binding peptides have elicited strong cytotoxic T cell responses in TAP1 -/- mice (Sandberg et al. 1996 and unpublished results). These results demonstrated that TAP1 -/- mice could mount strong CD8⁺ T cell responses against several independent MHC class I restricted peptides, but they do not exclude that some limitations in the functional CD8⁺ T cell repertoire may exist. As to the speculations on the role of class I bound peptides in positive selection (see e.g. Jameson et al. 1995), the results demonstrate that TAP-dependent peptides are not required for selection of a functionally diverse repertoire of CD8⁺ T cells (Sandberg et al. 1996). However, they do not exclude the possibility that the TAP-dependent peptides contribute to additional specificity and diversity.

DEVELOPMENT AND REACTIVITY OF CD8⁺ T CELLS IN TAP1/β2M -/- MICE

As described above, both TAP1 -/- and β2m -/- mice express low levels of MHC class I molecules and have few but detectable levels of mature CD8⁺ T cells. These results recently led us to explore whether a combined deficiency of TAP1 and β2m would give a more severe class I deficient phenotype than the one observed in ei-

TABLE III

Comparison of MHC class I expression, number and reactivity of CD8⁺ T cells in different MHC class I deficient mice.

	wild type	TAP1 -/-	β2m -/-	TAP1/β2m -/-
MHC class I expression	+++++ [1]	++	+	(+)
Number of $CD8^+$ T cells	+++++	++	+	(+)
Reactivity against allogeneic cells	+++++	++++	++	+
Reactivity against syngeneic (wild-type) cells	–	++	+++	+++

[1] The grading displayed in the Table is relative and is meant to illustrate the discussion in the text. For details see Ljunggren et al. 1995a and 1995b.

ther of the single mutant mice, and as a consequence of this a more dramatic impairment of the $CD8^+$ T cell repertoire.

To assess class I expression and function in the absence of both TAP1 and β2m, TAP1 -/- mice were crossed with β2m -/- mice to generate TAP1/β2m double mutant mice (Ljunggren et al. 1995a). Surface expression of $H\text{-}2K^h$ or D^h on cells derived from TAP1/β2m -/- mice was undetectable by immunofluorescence or immunoprecipitation, unlike what was observed in studies of TAP1 -/- and β2m -/- single mutant mice. Nonetheless, TAP1/β2m -/- cells were able to elicit a $CD8^+$ cytotoxic T cell (CTL) response in mice of different H-2 haplotypes and could be killed by anti-$H\text{-}2^h$ specific CTL. Furthermore, TAP1/β2m -/- skin grafts were rejected by bm1 mutant mice (Ljunggren et al. 1995a). Taken together, these results suggested that very low levels of conformed class I heavy chains could reach the cell surface even in the complete absence of TAP1 and β2m gene products. However, levels of MHC class I expressed were lower, by all criteria assessed, than those expressed on cells derived from the single mutant mice.

The inference that TAP1/β2m -/- cells must express some conformed class I heavy chains at the cell surface raised the possibility that some $CD8^+$ T cells may be selected in the thymus of TAP1/β2m -/- mice. Indeed, very low numbers of $CD8^+$ T cells were observed in peripheral blood lymphocytes from TAP1/β2m -/- mice. Levels were lower than those observed in either TAP1 -/- or β2m -/- mice. Nonetheless, TAP1/β2m -/- mice generated a surprisingly strong MHC class I specific $CD8^+$ T cell response after immunization and restimulation with allogeneic splenocytes (Ljunggren et al. 1995a). As was observed for alloreactive $CD8^+$ T cells from the TAP1 -/- and β2m -/- single mutant mice (Glas et al. 1994, Aldrich et al. 1994), alloreactive TAP1/β2m -/- $CD8^+$ T cells also cross-reacted with target cells expressing normal levels of $H\text{-}2^h$ class I molecules, but not with cells devoid of normal class I $H\text{-}2^h$ expression. When immunized and restimulated with cells expressing normal levels of $H\text{-}2^h$ class I MHC molecules, TAP1/β2m -/- effectors also readily killed $H\text{-}2^h$ expressing targets while class I deficient targets were spared. Notably, the anti-$H\text{-}2^h$ specific response of TAP1/β2m -/- mice was signifi-

cantly stronger than that of TAP1 -/- mice and at least as strong, or some times even stronger, than in β2m -/- mice (summarized in Table III). In line with the results from β2m -/- mice, TAP1/β2m -/- mice were also able to reject TAP1/β2m +/- skin grafts (Ljunggren et al. 1995a). In contrast to the success in generating allospecfic T cell responses in TAP1/β2m -/- mice, all attempts to generate MHC class I restricted responses in these mice have failed (unpublished observations). These studies include virus infections and peptide immunizations. In this respect, the TAP1/β2m -/- mice differ from the corresponding single mutant mice. These mice may thus serve as a model largely (if not totally) devoid of MHC class I restricted T cell responses.

In conclusion, the state of class I deficiency in the TAP1/β2m -/- mice is the most profound described thus far. Nonetheless, TAP1/β2m -/- mice appear to express minute amounts of free class I heavy chains and clearly appear to be able to select very low numbers of CD8⁺ T cells with functional properties similar, but not identical, to those of β2m -/- and TAP1 -/- mice (summarized in Table III).

ALTERED REACTIVITY OF THE CD8⁺ T CELL REPERTOIRES IN MHC CLASS I DEFICIENT MICE – MODELS AND INTERPRETATIONS

The available data suggest that the CD8⁺ T cells in TAP1 -/-, β2m -/- and TAP1/β2m -/- mice have been positively selected on the low levels of MHC class I molecules expressed in these mice. It is likely that positive as well as negative selection is affected by the low ligand density of class I molecules, and that the combination of these events will affect the final outcome of the selection process. In the present section we will discuss some of the arguments for selection on residual class I molecules in the TAP1 -/-, β2m -/- and the TAP1/β2m double mutant mice, but also mention some other explanations. The different interpretations are not mutually exclusive.

Selection of CD8⁺ T cells on other ligands than "classical" MHC class I molecules?

A question often raised is whether CD8⁺ T cells in TAP1 -/-, β2m -/- and TAP1/β2m -/- mice could have been selected on I-A^b (class II) molecules, or if they could represent a small set of CD8⁺ T cells that differentiates independently of class I recognition? Neither of these interpretations can be excluded, at least not for a minor population of the residual CD8⁺ T cell population in these mice. Nor can it be excluded that positive selection of some CD8⁺ cells could have occurred on class I like molecules such as CD1 or on class Ib molecules. For some subpopulations this may indeed be the most likely explanation (see Raulet 1994). However, several observations make it likely that (a majority of) the CD8⁺ T cells have been selected on low levels of MHC class I molecules, expressed independently of β2m and/or TAP-dependent peptides: (i) The class I molecules expressed on TAP1 -/-, β2m -/-

and TAP1/β2m -/- cells are sufficient to allow CD8⁺ CTL recognition (Vitiello et al. 1990, Bix & Raulet 1992, Glas et al. 1992, Hogquist et al. 1993, Ljunggren et al. 1995a), and might thus likewise suffice for positive selection. (ii) CD8⁺ T cells from all class I deficient mice show a strong bias towards reactivity with syngeneic class I H-2^h molecules (Glas et al. 1994, Aldrich et al. 1994, Ljunggren et al. 1995a,b). Class II -/- target cells of the H-2^h haplotype are killed as efficiently as class II +/- or +/+ target cells (Ljunggren et al. 1995a). (iii) Class II negative β2m -/- mice still display residual CD8⁺ CTL reactivity (Grusby et al. 1993). (iv) The number of CD8⁺ T cells differ in all mice (with highest levels in the TAP1 -/- mice and lowest levels in the TAP1/β2m -/- mice), while expression of I-A^h is not affected (H.G. Ljunggren and L. Van Kaer, unpublished results). This observation correlates well with levels of class I molecules expressed in the different mutant mice, being highest in the TAP1 -/- mice and lowest in the TAP1/β2m -/- mice (Ljunggren et al. 1995a, Ljunggren & Van Kaer 1995; illustrated in Table III).

The nature of selecting ligands in MHC class I deficient mice

What is the nature of the tentative selecting ligands in the different class I deficient mice? Cells devoid of β2m express a limited number of class I heavy chains, at least some of which are likely to be occupied by peptides (Glas et al. 1992, Machold et al. 1995). However, to our knowledge, there has been no detailed analysis of the peptide content of class I heavy chains in cells lacking β2m. Cells devoid of TAP1 are likely to express a limited number of class I heavy chains complexed with β2m, which could either be devoid of peptide, or occupied by peptides that are delivered to the class I molecules independent of the TAP complex (Ljunggren et al. 1990, Van Kaer et al. 1992). The situation is slightly different in TAP1/β2m double mutant mice where no biochemically or serologically detectable levels of class I molecules could be detected at the cell surface. Yet, indirect evidence points towards the existence of very low levels of conformed class I heavy chains that reach the cell surface in the complete absence of TAP1 and β2m gene products (Ljunggren et al. 1995a).

Why do CD8⁺ T cells in MHC class I deficient mice react against "self" class I expressed at a normal ligand density?

What mechanisms can explain the strong CD8⁺ T cell reactivity in the class I deficient mice against syngeneic MHC class I molecules expressed at a normal ligand density? In studies of β2m -/- mice by Glas et al., a point was made with respect to the requirement of a strong TCR affinity for "self" MHC class I as a prerequisite for positive selection in the β2m -/- mice (Glas et al. 1994). It was speculated that only the T cells with receptors having the (very) highest affinity for self class I meet the requirements for positive selection in the β2m -/- environment. Due to the low ligand density of class I molecules, few (if any) of these positively selected T

cells would meet the requirements for subsequent negative selection. $CD8^+$ T cells with high self affinity that would have been eliminated by negative selection in normal mice would here slip through. The net result of the selection event in the MHC class I deficient mice is a T cell repertoire with strong bias towards the MHC class I alleles expressed by the autologous host. However, this repertoire is not "harmful" in the autologous host where the levels of class I molecules in the periphery are to low to elicit autoaggression. The question is, is it "useful"? As reviewed above, there is no clear-cut evidence indicating that $CD8^+$ T cells in the β2m -/- mice mediate resistance in a way that would lead to e.g. clearance of a virus infection. Yet, it cannot be excluded that the (limited) pool of $CD8^+$ T cells may, at least to some extent, be useful in the class I deficient host. Future studies are required to investigate whether $CD8^+$ T cells selected in a β2m -/- environment are more useful in that environment than corresponding numbers of $CD8^+$ T cells that have been selected in a normal host and adoptively transferred to β2m -/- mice.

In addition to the effects suggested to be imposed by the low ligand density, qualitative effects could also explain the bias towards reactivity against cells expressing normal levels of syngeneic class I molecules (Glas et al. 1994, Aldrich et al. 1994, Ljunggren et al. 1995a,b, Cook et al. 1995). The latter argument has, in particular, been used to explain some of the "anti-self" class I reactivity in the TAP1 -/- mice (Aldrich et al. 1994), and has also been used to explain some of the observations by Cook et al. using β2m -/- mice of the $H\text{-}2^d$ haplotype (Cook et al. 1995). The MHC class I molecules expressed in the TAP1 -/- mice must be devoid of TAP dependent peptides. While these molecules still can select a pool of $CD8^+$ T cells, it is unlikely that T cells reactive with TAP-dependent peptides will be negatively selected in the TAP1 -/- mice. The absence of negative selection of $CD8^+$ T cells reactive with class I molecules loaded with TAP-dependent peptides can thus also be used to explain the bias towards reactivity to cells expressing normal levels of syngeneic class I. This reasoning can also be used for the β2m deficient mice (Glas et al. 1994, Cook et al. 1995), though we know significantly less about the peptide cargo of the class I molecules expressed at the cell surface in these mice. However, it is not unlikely that free class I heavy chains may pick up an, at least partially, different peptide repertoire than similar class I heavy chains that are associated with β2m. The extreme variant of this model is that the "self" reactivity is due only to peptides presented by non-mutant cells, and not at all related to ligand density.

It is important to spell out that neither of the above mentioned models are mutually exclusive. Rather, it is likely that both mechanisms may operate in the TAP1 -/- as well as the β2m -/- and the TAP1/β2m -/- mice. Future studies involving affinity measurements of the TCR, as well as models with mice expressing different levels of class I molecules loaded with the same set of peptides or mice expressing similar levels of class I molecules loaded with different sets of peptides may shed further light onto these issues. This is important when T cells of MHC class I deficient mice are used to ask basic questions about MHC recognition as described below.

MHC class I deficient mice as useful tools in the analysis of "self bias" in the CD8⁺ T cell repertoire

The observations reviewed here do not merely represent an esoteric demonstration of self bias in T cell selection under conditions where the flexibility of the repertoire is challenged to the extreme. The TAP1 -/- as well as the β2m -/- T cell repertoires may also provide useful experimental tools. Although it is widely accepted that positive selection must bias the repertoire for binding to the self MHC class I molecules irrespective of their peptide content, this has been difficult to demonstrate and analyze. Conventional T cells often show heteroclitic cross-reactivity on allogeneic MHC (see e.g. Nahill & Welsh 1993), but they do not kill or otherwise react against cells expressing self MHC. The ability of restricted T cells to recognize the MHC molecules as such cannot be assessed in conventional models. Using TAP1 -/- and β2m -/- mice, it may now be possible to study how MHC recognition varies between clones within an immune response or between different responses. The degree of MHC recognition during development of an immune response and subsequent establishment of memory can be analyzed. The ability of normal self MHC complexes to act as altered ligands and their ability to induce anergy or partial responses can be studied. The T cell repertoire in mice with defects in the MHC class I antigen presenting pathway may also be helpful in attempts to generate responses to cells with extremely low ligand densities of antigen/MHC complexes. This may be relevant in tumor immunology and in transplantation immunology. Taken together, the CD8⁺ T cells in the TAP1 -/- and β2m -/- mice may thus serve as a tool to analyze the "self" MHC bias of the T cell repertoire, a somewhat unexpected development for the first "MHC class I deficient" mice.

DEVELOPMENT AND REACTIVITY OF CD8⁺ T CELLS IN LMP2 -/- AND LMP7 -/- MICE

Several lines of evidence indicate that a large proteolytic complex, termed the proteasome, is involved in the cleavage of endogenous proteins to smaller peptide fragments (reviewed in Goldberg & Rock 1992). Such peptides, largely thought to be generated in the cytosol, are subsequently translocated into the ER where they take part in the assembly of MHC class I molecules. Two subunits of the proteasome, termed LMP2 and LMP7, have been of particular interest since they are encoded within the MHC gene complex (Martinez & Monaco 1991, Brown et al. 1991, Glynne et al. 1991, Kelly et al. 1991, Ortiz-Navarrete et al. 1991). The involvement of the LMP2 and LMP7 gene products in the generation of antigenic peptides recognized by CD8⁺ T cells has more recently been studied with mutant cell lines (Momburg et al. 1992, Yewdell et al. 1994, Zhou et al.1994, Cerundolo et al. 1995, Sibille et al. 1995) as well as with cells from LMP2 -/- (Van Kaer et al. 1994) and LMP7 -/- (Fehling et al. 1994) mice. Absence of proteasome subunits such as LMP2 and LMP7 have a pronounced effect on the presentation of some an-

tigenic peptides (Fehling et al. 1994, Cerundolo et al. 1995, Sibille et al. 1995), while the effects are less pronounced for other peptide antigens (Momburg et al. 1992, Yewdell et al. 1994, Zhou et al. 1994).

LMP2 -/- and LMP7 -/- mice, respectively, were generated by two different groups almost simultaneously (Van Kaer et al. 1994, Fehling et al. 1994). The two mutant strains showed at least partly different phenotypes. In the LMP2 -/- mice, no difference as compared to wild-type mice was observed with respect to cell surface MHC class I expression, while the LMP7 -/- mice showed a 25-40% reduction in expression of MHC class I molecules. However, LMP7 -/- mice had normal levels of $CD8^+$ T cells, while a 30–40 % reduction in numbers of $CD8^+$ T cells were seen in the LMP2 -/- mice. Cells from both mice showed limitations in their ability to present certain antigens to $CD8^+$ T cells (Van Kaer et al. 1994, Fehling et al. 1994). Proteasomes purified from the spleen and liver of LMP2 -/- mice showed altered peptidase activities (Van Kaer et al. 1994).

It is not entirely clear why LMP2 -/- mice have a reduction in numbers of $CD8^+$ T cells. It was speculated that these mice could express self peptides with a reduced complexity, which might result in reduced efficiency of $CD8^+$ T cell positive selection and reduced complexity of the T cell repertoire (Van Kaer et al. 1994). The spectra of TCR Vβ chains expressed did not markedly differ between LMP2 -/- and wild type mice, though this does not exclude differences in the T cell repertoire with respect to diversity. The observed reduction in $CD8^+$ T cell precursors against influenza virus observed in LMP2 -/- mice is interesting, but somewhat difficult to interpret with respect to the diversity and reactivity of the $CD8^+$ T cells. The differences observed could be caused by differences either in antigen presentation and/or in the T cell repertoire.

In conclusion, there is no evidence for dramatic alterations in the T cell repertoire in LMP2 -/- and LMP7 -/- mice. It is difficult to draw any detailed conclusions with respect to the diversity and specificity of the $CD8^+$ T cell repertoire in these mice. A more detailed study of their $CD8^+$ T cell repertoire would certainly be of interest.

THE ROLE OF MHC CLASS I PRESENTED PEPTIDES IN POSITIVE SELECTION AS STUDIED IN FETAL THYMIC ORGAN CULTURES

Since peptides are an integral part of the MHC molecules, it is likely that the MHC molecules which are recognized by the TCRs of immature thymocytes during both positive and negative selection are occupied by self-peptides. Several studies have implicated peptide specificity in the process of T cell positive selection (Nikolic-Zugic & Bevan 1990, Jacobs et al. 1990, Sha et al. 1990, Ohashi et al. 1993, Hogquist et al. 1993, 1994, Ashton-Rickardt et al. 1993, 1994, Sebzda 1994, Jameson et al. 1994, Hogquist et al. 1995). Mutations in the antigen binding grooves of both $H\text{-}2K^b$ and D^b molecules were shown to affect positive selection in TCR transgenic mice (Nikolic-Zugic & Bevan 1990, Jacobs et al. 1990, Sha et al. 1990,

Ohashi et al. 1993). One possible explanation for this effect is that mutant class I molecules may present a peptide repertoire different from that presented by wild-type molecules to T cells that are being selected in the thymus.

More recent studies with β2m -/- or TAP1 -/- fetal thymus organ cultures (FTOC), an *in vitro* system for studies of thymic selection of T cells, have convincingly demonstrated that specific peptides may affect positive selection of $CD8^+$ T cells (Hogquist et al. 1993, 1994, Ashton-Rickardt et al. 1993, 1994, Sebzda 1994, Jameson et al. 1994, Hogquist et al. 1995). Briefly, single peptides, when added to the organ cultures from TAP1 -/- thymi, were able to reconstitute surface expression of class I molecules on thymic epithelial cells. However, only a subset of these peptides was able to induce positive selection of $CD8^+$ T lymphocytes. Furthermore, more complex mixtures of peptides were very effective at inducing T cell positive selection while being rather inefficient in stabilizing class I cell surface expression. More recent data, relying on crosses between different TCR transgenic mice and β2m -/- and TAP1 -/- mice, respectively, have strengthened the notion of peptide specificity in positive selection (Hogquist et al. 1994, Ashton-Rickardt et al. 1994, Ashton-Rickardt & Tonegawa 1994, Sebzda 1994, Jameson et al. 1994, Hogquist et al. 1995, Jameson et al. 1995). Taken together, these data have been used to argue that peptide not only serves to stabilize the class I structure during T cell positive selection but that it actually contributes to the specificity of this process. The data have also led to a debate on whether the nominal antigen *per se* can cause positive selection of a given T cell receptor. While Tonegawa and colleagues have data supporting positive selection on low concentration of the nominal antigen (see e.g. Ashton-Rickardt et al. 1994), Bevan and colleagues have argued that positive selection predominately occurs on antagonistic peptides rather than the nominal antigen (Hogquist et al. 1994, Hogquist et al. 1995). Irrespective of this discussion, these studies have demonstrated the usefulness of TAP1 -/- and β2m -/- mice in studies of the role of MHC class I presented peptide in T cell selection.

SUMMARY AND CONCLUDING REMARKS

The generation and characterization of β2m -/- and TAP1 -/- mice have underscored the role of MHC class I molecules in selection of a normal $CD8^+$ T cell repertoire. The mice have also given insights into the role of class I molecules in the development of other T cell subsets. In contrast to what was initially reported, β2m -/- as well as TAP1 -/- mice express low levels of class I molecules at the cell surface and select a small pool of mature $CD8^+$ T cells. Studies of these mice have demonstrated that potent biological responses can be elicited from a relatively limited pool of pre-existing $CD8^+$ T cells. These include MHC class I restricted and allospecfic responses, including rejection responses against large tumor grafts. Interestingly, the $CD8^+$ T cells selected in the class I deficient mice differ from similar cells in wild-type mice in that they show a strong bias towards reactivity with syngeneic class I molecules expressed at a normal ligand density such as in wild-type

or H-2 syngeneic mice. This reactivity may be a direct effect of T cell selection on low ligand density MHC class I and/or limitations in the MHC class I presented peptide repertoire, as would be predicted from recent affinity and avidity models for T cell selection. The fact that CD8⁺ T cells selected in these mice may react with "self" MHC class I expressed at a normal ligand density could be of use in studies of TCR affinity to self MHC, e.g. in changes in TCR-MHC recognition during immune responses and development of memory. They may also be used in attempts to evoke immune responses to targets cells expressing very low levels of MHC class I molecules, a matter that is relevant in e.g. tumor immunology.

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Structure and Function of the TAP Protein and Related Ly-6-Linked Molecules[1]

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Introduction

T cell activation is major histocompatibility complex (MHC)-restricted and antigen-dependent. The specificity of activation is imparted by the T cell receptor/T3 complex, however, the molecular interactions occurring during receptor triggering, and the role of additional cell surface molecules is incompletely understood. Our laboratory has been investigating a family of Ly-6-linked proteins that participate in these events. In this report we review studies performed in our laboratory characterizing the structure and function of the T cell-activating protein (TAP) and TAPa molecules and related proteins.

Structure of the TAP and TAPa proteins

TAP is a T lymphocyte surface protein with an apparent molecular weight of 10–12 kd under nonreducing conditions and 15–18 kd upon reduction, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [1, 2]. The decrease in electrophoretic mobility upon reduction indicates the presence of intrachain disulfide bonds.

TAP migrates as three closely spaced bands when metabolically labeled [1]. All three species are alternate forms of the same protein. This is

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evident from their coordinate behavior upon reduction [2, 3], their common migration pattern in isoelectric focusing gels [3] and by comparative N-terminal amino acid sequencing [3]. In pulse chase experiments the middle molecular weight band is the earliest detectable species which is subsequently partially converted into a higher and lower molecular weight form [3]. The two higher molecular weight forms of TAP are expressed on the cell surface. This is apparent in surface ^{125}I-labeling experiments [2] and by their release upon surface enzymatic digestion [2]. The species of lowest molecular weight appears to be an internal form that may arrise from a proteolytic event.

TAP is removed from the cell surface by treatment with a phosphatidylinositol-specific phospholipase C [2]. Subsequently it is recovered in the supernatant in soluble form. It is therefore not a transmembrane protein but is anchored to the cell surface via a phosphatidylinositol lipid linkage [2, 4].

TAP is a glycoprotein. All three TAP bands specifically label with [^{3}H]-glucosamine [2]. Tunicamycin treatment or peptide N-glycanase digestion, which inhibit synthesis of, and remove N-linked sugars, respectively, fail to alter the migration of TAP on SDS-PAGE [3]. Treatment with trifluoromethane sulfonic acid decreases the mobility of the upper TAP band and monesin inhibits the biosynthesis of this species [3]. Therefore the upper band arises by the addition of O-linked sugars in the Golgi complex. The two lower molecular weight species of TAP have minimal carbohydrate. This may be present, in part, in the lipid-glycosidic linkage [4].

The TAPa protein is structurally distinct from TAP: First, it has an apparent molecular weight of 15–16 kd, unaffected by sulfhydryl reduction [1–3]. Second, it does not label in ^{125}I-lactoperoxidase-catalyzed reactions, although it is expressed on the cell surface [1]. Third, the molecule appears to be nonglycosylated [2].

When analyzed by flow fluorocytometry it is removed from the cell surface by a phosphatidylinositol specific phospholipase C [2]. Thus both TAP and TAPa might be anchored to the plasma membrane via the same or a very similar linkage.

TAP and TAPa appear to be related on the T cell surface. Monoclonal antibodies (MAb) against the respective molecules compete for binding to the T cell surface [1]. Furthermore these two molecules specifically comodulate, and coimmunoprecipitate [5]. These findings suggest that TAP and TAPa form a complex on the cell surface. As discussed below these two proteins are related genetically and in their expression.

Function of the TAP Molecule

Antibody cross-linking of the TAP molecule on T lymphocytes results in profound cellular activation [1, 6]. This effect is highly specific and is not observed with antibodies against most other cell surface antigens (eg. L3T4, Lyt-2, LFA-1, or H-2) [1, 6]. For T-T hybrids, anti-TAP MAb is sufficient to directly stimulate maximal lymphokine production, when added in soluble form, in nanogram amounts [1]. TAP cross-linking is required, as Fab′ monovalent antibody is inactive until cross-linked [7]. However, no additional interacting cells or exogenous second signals are required [1]. Not all anti-TAP MAb are directly stimulatory [1]. However, these latter antibodies become activating if further cross-linked [7]. From these observations, it appears that anti-TAP cross-linking is capable of stimulating all necessary activation signals. One of these signals that has been directly measured is a rapid increase in intracellular free calcium [7]. This effect is specific as it is not observed with control antibodies. Surprisingly, this latter response requires more stringent conditions than are required for activation [7]. The basis for this discrepancy is unknown and requires further study. Similarly, the nature of other signals generated upon TAP cross-linking is unknown.

Resting heterogeneous T cells are also specifically stimulated by the activating anti-TAP MAb to enter cell cycle, if appropriate second signals are present (e.g. IL 1 or phorbol esters) [1, 7]. This response is mediated by an autocrine pathway, wherein IL 2 (and possibly BSF-1) is produced and lymphokine receptors are expressed [7]. This parallels activation through other cell surface receptors and raises the possibility that these molecules are involved in a common pathway of activation.

Three families of murine T cell surface proteins are known to be stimulatory upon antibody cross-linking: the TCR/T3 complex [8]; TAP and related Ly-6 proteins (discussed below); and Thy-1 [9]. It is of interest that both TAP and Thy-1 are anchored to the membrane via phosphatidylinositol [2, 10]. This observation raises the possibility that this linkage could be involved in signal transduction [2]. Inositol phospholipids have been implicated in the generation of receptor second messengers [11]. Alternatively, this linkage could facilitate protein translocation within the plasma membrane [12].

With the exception of the TCR/T3 complex, the physiologic role of the activating cell surface proteins is unknown. Three sets of observations suggest that TAP may participate during immune specific activation of T

cells. First, antibody cross-linking of TAP molecules greatly increases the stimulus-response pattern of antigen activation, even at levels of TAP cross-linking that are themselves insufficient to cause activation [1]. Second, a nonactivating anti-TAP MAb interferes with antigen-specific immune stimulation [1]. Third, preliminary analysis of T-T hybrids with selective defects in TAP expression demonstrate impaired activation characteristics [unpublished data]. Taken together these results suggest that TAP molecules participate in immune activation.

Expression of TAP and TAPa Molecules

TAP is not detectable on T cell-depleted bone marrow [13]. It is first detected intrathymically on a subpopulation of early fetal or adult immature (L3T4- and Lyt2-) thymocytes [6]. It is active on these cells. TAP is not detectable on cortical (Lyt2+, L3T4+, TL+, J11D+ and PNA+) thymocytes [6]. These latter cells account for approximately 85% of adult thymocytes and are functionally immature. TAP is expressed on the majority of medullary (L3T4+ or Lyt2+) thymocytes [6]. It is functional on these latter cells and its expression defines the immunocompetent compartment. Thus the small number of TAP-positive cells account for responsiveness to polyclonal mitogens and alloantigens [6]. It is possible therefore that the expression of TAP is directly related to functional maturation.

On resting lymphocytes TAP is primarily expressed on T cells [1, 14]. It is expressed on all L3T4-positive cells and 30–50% of Lyt2-positive cells [14]. The heterogeneity of this latter subset is in part attributable to differences in expression on cytotoxic T lymphocyte (CTL) precursors and effectors [14]. In this context, it is of interest that lymphocyte activation is one parameter that effects TAP expression [14]. TAP expression is markedly increased on activated T cells and it is synthesized by activated B cells [1, 14]. Given the function of TAP, it is conceivable that these differences in expression will affect the activation characteristics of these respective cells.

In all cases TAP and TAPa are coexpressed. This could reflect common gene regulation (see below) and/or protein association (see above). Recent analysis of TAP expression mutants has demonstrated coordinate loss in surface expression of these two molecules [5]. Biosynthetic analysis of this defect reveals an absence of TAP, while the TAPa protein is synthe-

sized. This suggests that TAP is required for the surface expression of TAPa [5]. This behavior is similar to that observed for other cell surface-associated proteins [15, 16].

Genetics of TAP and TAPa: Linkage to the Ly-6 Locus

Both TAP and TAPa have an allelic polymorphism in their expression that is detected serologically. By an analysis of the strain distribution of TAP in inbred and recombinant inbred and congenic murine strains, a genetic linkage to the Ly-6 locus was defined [13]. The strain distribution pattern of TAP and TAPa matches exactly that of the b allele of the Ly-6 locus.

This locus was first defined by McKenzie et al. [17] and also Feeney and Hämmerling [18] using murine alloantisera that were thought to detect a simple allelic polymorphism consisting of 2 alleles, Ly-6.1 (Ly-6^a) and Ly-6.2 (Ly-6^b). Studies of Ly-6 cell expression and serology using MAb suggest the existence of 7 or more distinct epitopes, although it is unclear whether all of these distinctions are meaningful [19–23]. The Ly-6 antigens are varyingly expressed on a variety of hematopoietic cells, including on T lymphocytes, as well as on at least some other tissues.

What is the molecular complexity of this locus and the relationship of TAP/TAPa to other Ly-6 antigens? We find evidence for at least two distinct families of Ly-6-linked proteins. The first is made of 10–12 kd internal disulfide-bonded proteins. This includes TAP and Ly-6.1E. These two antigens are distinct on SDS-PAGE [3] and in tissue distribution [1, 22]. There is genetic evidence reported for a third antigen of this class, that is expressed solely on bone marrow [22]. The second class consists of 15–16 nondisulfide-bonded proteins. This includes TAPa and 34.2.11 that are indistinguishable on 2-D IEF/SDS-PAGE gels [13] but are clearly serologically distinct, and H9/25 [13]. An additional antigen with distinct tissue distribution has been described that may fall into this category (ThB) [21]. These results are consistent with recent findings in other laboratories [23–25]. In addition, there are several antigens detected by anti-Ly-6 MAb whose molecular characteristics are less well defined. For example, there have been reports of 33 kd Ly-6 proteins [21]. However, for one such protein, Ly-6.2A (defined by the S.8.106 MAb), discrepant results have been reported [25]. Therefore, the relationship between these Ly-6 molecules and the two families of Ly-6 proteins described above requires fur-

ther study. Recently LeClair et al. [26] as well as our laboratory [unpublished results] have isolated cDNA clones of Ly-6-linked genes that demonstrate considerable complexity on Southern blots of genomic DNA, suggesting that the Ly-6 locus contains a multigenic family.

Based on the analysis of TAP and TAPa discussed above it seems likely that several Ly-6 molecules will be anchored via phospholipids although this remains to be formally demonstrated.

We have performed comparative studies of the cellular expression of Ly-6 antigens with several points of interest. First, there is a complex developmental regulation of Ly-6 antigen expression [2, 6]. Virtually every stage of T lymphocyte ontogeny has a unique pattern of antigen expression. Second, lymphocyte subsets also vary in expression of these molecules [14]. Third, the display of these molecules can be altered upon lymphocyte activation, as noted above for TAP [14]. Finally, the expression of these antigens does not appear to be mutually exclusive [2].

The function of the Ly-6 molecules had been largely unknown. Flood et al. [27] reported that the HD-42 MAb, originally produced against the Meth A sarcoma and detecting an Ly-6-linked antigen (Ly-6.1E), interferes with the function of resting and activated T cells, although this has not been reproduced [28]. The TAP protein, however, clearly assigned a function to an Ly-6-encoded protein. Given this finding it is clearly of interest to determine whether other distinct Ly-6 molecules have similar activating potential. If MAb against such proteins are added to culture they do not, by themselves, activate either normal T cells or T-T hybrids [2, 7]. However, Malek et al. [28] and Yeh et al. [7] have observed that if these antibodies are bound to T cells and are then further cross-linked, they are capable of stimulating these cells. Currently this analysis has been restricted to those antigens expressed on T lymphocytes. Given the distribution of Ly-6 molecules, it is possible the role of TAP-like proteins will extend to other cell lineages and tissues.

Concluding Remarks

TAP has a number of features that make this protein of particular interest and that clearly warrants its further study. It is one of the few T lymphocyte membrane molecules capable of stimulating upon cross-linking and appear to do so through a pathway similar to other T cell surface receptors. Furthermore, this molecule may play a significant role in phys-

iologic T cell activation. Anti-TAP MAb can modulate antigen-specific T cell activation and TAP expression mutants display activation defects.

Given the findings above, it is of interest that this functional molecule is not a transmembrane protein but is linked to the membrane via a rare phospholipid linkage. This linkage is shared by at least one other activating protein (Thy-1), thus raising the possibility that this anchorage is of considerable functional importance.

On unstimulated lymphocytes, TAP is expressed on T cells and defines additional heterogeneity within the major T cell subsets. Its profile of expression is rapidly altered upon cell activation. These features are intriguing given the functional role of this molecule. Furthermore, ontologically, TAP is first detected in the thymus, where it is expressed on only a small percentage of cells. TAP expression, however, defines the immunocompetent compartment of the thymus and thus could be involved in this functional maturation.

Finally, the genes controlling TAP and TAPa expression are tightly linked to the Ly-6 locus. There is molecular evidence for two families of 'homologous' Ly-6 proteins, which all have activating potential where examined. This family of poteins appears to be more widely distributed on a variety of cell types and developmental stages. Thus the role of TAP-like proteins might extend to a number of cell systems.

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T cell selection and autoimmunity: flexibility and tuning

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Peptide–MHC interactions govern the fate of T cells in the thymus and the peripheral T cell repertoire. Recent progress has involved investigating how different peptides influence T cell selection and mature T cell function and the subsequent implications for tolerance and autoimmunity.

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Abbreviations

APC	antigen-presenting cell
β_2m	β_2-microglobulin
Hb	hemoglobin
hsp	heat shock protein
Ii	invariant chain
LCMV	lymphocytic choriomeningitis virus
Mls	minor lymphocyte stimulatory
SEB	staphylococcal enterotoxin B
TCR	T cell receptor

Introduction

Selection in the thymus imposes a delicate tripartite balance: it allows the generation of useful T cells that are restricted to self-MHC; it prevents the maturation of T cells that would be most efficient in inducing an autoimmune response; and, it preserves a repertoire that has the ability to respond to a wide array of foreign antigens.

Positive selection permits the survival of thymocytes that express TCR $\alpha\beta$ heterodimers with low affinity/avidity for self MHC ligands [1]. This process occurs at the double-positive ($CD4^+CD8^+$) stage of T cell development, through interactions with cortical epithelial cells, and progresses to single-positive $CD4^+$ or $CD8^+$ T cells [2]. Negative selection tolerizes T cells that have potential reactivity to self-ligands that are presented at adequate levels in the thymus by inducing clonal elimination or unresponsiveness. This occurs at the double-positive or single-positive stage, primarily through interactions with bone marrow derived cells.

To understand how a discriminatory T cell manouvres its way through these selection pathways, it is important to identify interactions that permit positive selection, negative selection and antigen-specific effector function.

Affinity/avidity model of thymocyte selection

Through the years many models have addressed the mechanisms that lead to positive or negative selection in the thymus. The majority of the evidence obtained supports the affinity/avidity model of thymocyte development [1] (for discussion of other models see [3–6]. According to this model, if an immature thymocyte cannot interact with ligands expressed by thymic epithelial cells, the thymocyte is not rescued from programmed cell death. Low-affinity/avidity interactions result in sufficient signalling to upregulate genes necessary for survival of the thymocyte, while high-affinity/avidity interactions lead to cell death.

Although the basic model predicts that the affinity between the TCR and the peptide–MHC complex and the total avidity between the T cell and the stromal cell are the crucial parameters influencing the outcome of T cell development, other factors must also be considered. By definition, affinity constants reflect the association and dissociation rates between the interaction of two soluble ligands. Because development deals with cell–cell interactions, coreceptors, costimulatory, adhesion and signalling molecules should also be considered. For TCR interactions, other parameters besides affinity will affect the outcome of selection, such as the stability of the peptide–MHC complex, the effect of aggregation of the receptor on the formation of a signal transduction complex, and whether the 'off' rates are compatible with complex formation and the overall kinetics of the surface molecule interactions [6–9]. Recent experiments bring new insights into interactions required for T cell development, and support a basic affinity/avidity model.

Defined peptides mediate positive and negative selection

Several studies have examined the influence of defined peptides in T cell selection. Using a combination of genetically altered mice, MHC class I restricted TCR transgenic mice were generated that did not express either β_2-microglobulin (β_2m) or TAP (transporter associated with antigen processing), both of which are required for stable expression of MHC class I molecules on the cell surface. In the absence of normal class I expression, the transgenic TCR^+ thymocytes did not mature *in vivo* or in fetal thymic organ cultures. Cocultivation of these thymic lobes with different concentrations of defined peptides demonstrated that ligands related to the original peptide antigen recognized by the transgenic TCR were able to promote positive selection [10–13,14••,15••]. These studies also showed that low concentrations of related peptide ligands could promote positive selection whereas higher concentrations of the same peptide would promote deletion [10,11,12]. Therefore, both qualitative and quantitative properties of the peptides could define selection events in the thymus, and were consistent with the affinity/avidity model.

One discrepancy has been demonstrated in these models that have examined the properties of the peptides involved in thymic selection. Studies using the ovalbumin-specific TCR transgene have concluded that antagonist peptides, which do not elicit a response from the mature transgenic T cells, were most efficient in promoting positive selection of functional T cells [10,13,14••,16]. In contrast, using the lymphocytic choriomeningitis virus (LCMV)-specific TCR transgenic model, agonist peptides could promote the maturation of CD8+ transgenic T cells [11,12,15••] that responded to high-affinity ligands [15••]. Since every TCR may interact with the peptide–MHC complex in a slightly different orientation, each receptor may rely on a different number of contact points on the peptide or the MHC molecule. In the case of the ovalbumin-specific transgenic TCR model, more affinity may be contributed by contact points with the MHC. Therefore, relatively less affinity would be required by contact points through the peptide to achieve the desired threshold for positive selection. Consequently, antagonist peptides would be able to promote positive selection. The LCMV-specific transgenic TCR may be more dependent upon contact points with the peptide and require agonist peptides to provide sufficient interactions to maintain signals for positive selection. It may not be relevant, therefore, whether the peptide is an agonist or an antagonist: it may be the overall interaction of a given TCR with peptide–MHC that is sensed by the T cell and which dictates the consequences of thymocyte selection.

Using surface plasmon resonance, Gascoigne's group [17••] has directly examined the affinity between the ovalbumin-specific TCR and the peptide–MHC that leads to positive selection or negative selection. Using TCR–peptide–MHC interactions that have been shown previously to mediate defined selection events in fetal thymic organ culture, this analysis has shown a correlation between low affinity and positive selection and between high affinity and negative selection, lending full support for the affinity/avidity model.

Flexibility in positive selection

Although these models have provided clues to the peptide ligands and mechanisms that define the opposing events of positive and negative thymocyte selection, they do not necessarily imply that interactions required for positive selection are highly specific or rigid. If there is a relatively 'strict' recognition for positive selection, then millions of peptides must be present in the thymus to mediate the maturation of a vast T cell repertoire. This is not feasible. It is likely that the thymic epithelial cell is presenting a similar image of peptides to that presented by other cells [18–20], possibly providing a peptide spectrum including 2000 different epitopes [21]. This would not allow for the required diversity of T cells if positive selection was defined by precise interactions.

Experimental evidence demonstrates that a relatively normal spectrum of Vβ genes was found if CD4+ or CD8+ T cells are selected in the presence of a single peptide or with limited peptide diversity [22,23•,24••,25•]. In addition, experiments using fetal thymic lobes from the defined 2C transgenic TCR (H-2K^b-restricted, alloreactive to L^d) β_2m-deficient mice have demonstrated that unrelated peptides were capable of mediating positive selection [26•]. These studies illustrate that interactions leading to positive selection are flexible.

The role of the peptide is not simply to stabilize the MHC molecules [3] because some degree of specificity exists. This has been shown using fetal thymic organ cultures from TCR transgenic animals that are unable to naturally process class I molecules. Addition of peptides that are known to stabilize the relevant MHC molecules but do not interact with the transgenic TCR does not mediate positive selection of transgenic T cells [10,11,15••,26•]. Positive selection has also been examined for CD4+ T cells in the absence of the invariant chain (Ii). Because the invariant chain is required for normal MHC class II assembly and intracellular trafficking, Ii-deficient mice express reduced levels of class II molecules with an atypical form that has an altered spectrum of peptides. In an Ii-deficient background, two out of three class II-restricted transgenic TCRs failed to undergo efficient positive selection [27•]. Similarly, other models with limited class II peptide diversity, showed a reduction in CD4+ T cell maturation [25•,28•,29•].

Furthermore, if the peptide does not provide any specificity then receptors that have been restricted to the same MHC ligand should have the same selection 'requirements'. This is not the case. Two H-2D^b-restricted receptors the H-Y and LCMV models, have been bred with the D^b mutant H-2^{bm13} strain. The H-Y-specific TCR cannot be selected by bm13, whereas the LCMV-specific TCR is more efficiently selected by bm13 than H-2D^b [30,31]. Taken together, these data demonstrate that a degree of specificity is necessary for positive selection; however, considerable flexibility is permitted which is probably defined by the affinity/avidity threshold for positive selection.

Range of affinities for positive selection

Consistent with the idea that interactions that mediate positive selection are flexible, evidence suggests that a range in affinity/avidity can result in positive selection. Early experiments using TCR transgenic mice expressing class I variants of the selecting MHC molecules demonstrated that a few amino acid changes in the MHC class I molecule still resulted in positive selection of the defined transgenic TCR. The percentage of the selected transgenic T cells, however, was altered in the thymus and mature T cell repertoire [31,32]. This suggests that slight modifications in the

peptide–MHC expressed by the thymic epithelium results in a different efficiency of positive selection. In addition, affinity measurements using defined TCR–peptide–MHC interactions that permit positive selection *in vitro* have shown that there is a range in affinities that permits positive selection of a thymocyte expressing a defined TCR [17••].

Tuning selection and specificity by defining resting thresholds

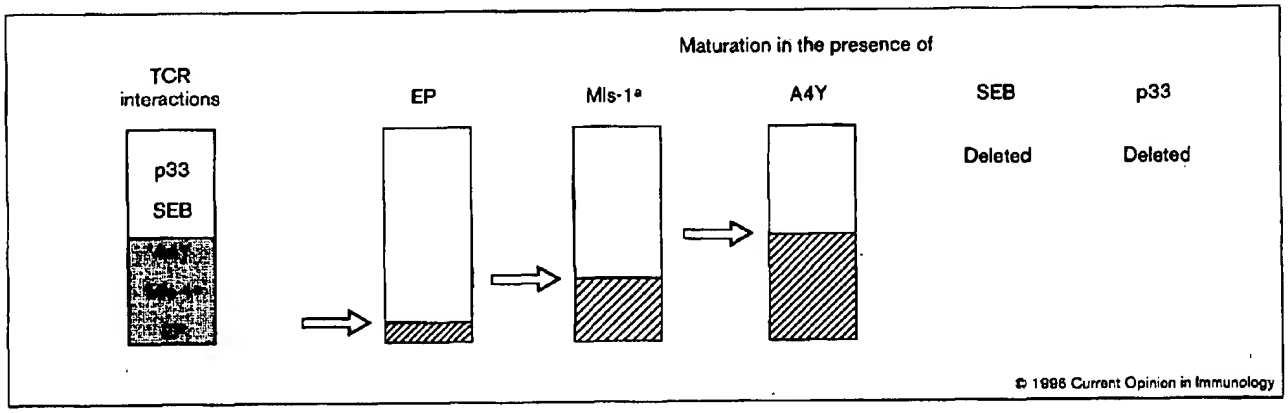
The existence of a range in affinity/avidity that is sufficient for positive selection raises several questions. Is there any change in the specificity of a T cell if positive selection occurs through interactions with the lowest or highest avidity ligands? For some TCRs, it is also possible that the maximum avidity interaction that will allow positive selection is not available to the T cell during interactions with cortical epithelium. Is there any potential for 'tuning' the T cell during interactions with bone marrow derived cells without causing clonal deletion?

To address these questions, experiments may be done with a 'monoclonal' cell population, since each T cell clone specific for a defined antigen potentially has different cross-reactive ligands [33,34••,35••]. Studies using the LCMV-specific TCR transgenic model (specific for peptide KAVYNFATC [single-letter code for amino acids], H-2D^b-restricted) have examined the effects of the lower-affinity peptide A4Y (KAVANFATM). Using fetal thymic organ culture from TCR transgenic β_2m-deficient mice, cocultivation with A4Y and exogenous β_2m led to an increase in transgenic TCR+CD8+ T cells, that were unable to respond to A4Y but could respond to a strong agonist ligand p33 (KAVYNFATM). TCR+CD8+ T cells maturing in the presence of the 'endogenous peptides' could mount a minimal response to A4Y, and an equally strong response to p33 Fig. 1 [15••]. Therefore, maturation of the transgenic TCR+ T cells in the presence of A4Y (rather than endogenous peptides) may have altered the 'resting threshold' for the T cell, eliminating the potential response to A4Y and reducing the spectrum of activating ligands for the mature T cell.

Using the same LCMV TCR transgenic model, other studies have addressed whether the presence of tolerizing ligands in the thymus alters the responsiveness of the T cell population. Because the LCMV-specific transgenic TCR uses Vβ8.1, which interacts with the superantigens minor lymphocyte stimulatory (Mls)-1^a and staphylococcal enterotoxin B (SEB) [36,37], the reactivity to three ligands was studied. By breeding the transgenic TCR mice (H-2^b) with CBA/J (H-2^k, Mls-1^a) or CBA/CaJ (H-2^k, Mls-1^b) mice, the responsiveness of T cells maturing in the presence or absence of the low-affinity ligand Mls-1^a was examined. Transgenic TCR+ T cells from H-2$^{b/k}$ Mls-1^a animals, proliferated in response to SEB and LCMV but not to Mls-1^a, whereas TCR+ T cells from H-2$^{b/k}$ Mls-1^b animals could respond to SEB, LCMV and weakly to Mls-1^a (Fig. 1) [38••]. Therefore, the presence of a tolerizing ligand during thymic selection could alter the spectrum of activating ligands in the mature monoclonal T cell population.

In summary, these studies suggest that TCR thymic ligand affinity is an integral component of T cell development and modification. T cell maturation in the presence of low-affinity ligands may set the resting threshold of thymocytes, which consequently defines

Figure 1



The 'window' of affinity available for T cell positive selection also reflects the range for T cell tuning. Ligands with different abilities to interact with the LCMV-specific transgenic TCR have been defined. The order for the TCR interactions is based upon physiological levels of the given ligand, or optimal concentrations of peptides as defined by proliferation assays. Maturation of the transgenic TCR in the presence of various ligands imposes different fates on the T cell. Arrows designate the resting threshold set under the different maturation conditions. Hatched areas reflect interactions that do not elicit a response in mature T cells. Open areas correspond to interactions capable of eliciting a T cell response. Shaded regions outline the range of interactions that may potentially involve T cell tuning. EP, endogenous peptide(s).

the spectrum of potential ligands for a given T cell. Since strong interactions during selection result in clonal deletion [39–41] (Fig. 1), there is a relatively narrow affinity range where the resting threshold may potentially be adjusted or tuned. Tuning may occur during both positive and negative selection by interactions with thymic epithelial cells or bone marrow derived cells. Coreceptor [13,42,43••], adhesion, and signalling molecules may be involved in this process. It is also likely that in order to maintain this resting threshold that is set during thymic ontogeny, ligands with a similar affinity must be present in the periphery. These events may reflect a tolerance mechanism known as clonal unresponsiveness, inactivation or anergy.

Flexibility in mature T cell interactions: implications for tuning

Traditionally, interaction with a given TCR has been defined as being highly specific for a combined peptide–MHC. Recent data have suggested, however, that not only do altered peptide ligands envoke various subsets of T cell responses, but that different ligands possessing either structural or minimal amino acid homology may generate a response (for review see [44]). By searching a protein database, Allen's group [34••] have identified peptides with minimal homology to the cognate antigen being investigated. These peptides were able to trigger apoptosis, or induce agonist or partial agonist function from a defined T cell clone. In addition, a human T cell clone specific for thyroid peroxidase has been reported to recognize two peptides that have almost identical electrostatic surfaces. These comparisons have been generated by computer modelling and suggest that cross-reactive T cell responses may involve peptides with 'similar antigenic surfaces' in addition to amino acid homology [45••]. Similarly, an I-A^d-restricted hybridoma specific for sperm whale myoglobin can be stimulated with five peptides containing overlapping sequences but different core residues [46•].

Given the emerging evidence that mature TCR recognition is flexible, it may also be necessary to 'tune' the resting threshold of a T cell to cross-reactive self ligands as a mechanism of peripheral tolerance. Studies by Vidal *et al.* [43••] may support the mechanism of peripheral T cell tuning to relatively low-affinity ligands. Using a TCRβ transgenic model, the influence of endogenous altered peptide hemoglobin (Hb) ligand was investigated. Evidence suggested that the transgenic T cells had been selected to ignore endogenous levels of Hb. If the level of Hb peptide presentation was increased by transfection of APCs, however, antagonist effects could be detected

Table 1

Cross-reactive ligands associated with autoimmunity.*

T cell/protein	Peptide																Reference
Mouse CD4+, I-A^{q7}-restricted																	
†Glutamic acid decarboxylase	A	R	Y	K	M	F	P	E	V	K	E	K	G	M	A	A	[57]
Coxsackie virus P2-C	L	K	V	K	I	L	P	E	V	K	E	K	H	E	F	L	
Human CD4+ clone, HLA-DQ1-restricted																	
†Myelin basic protein (85–99)	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R		[35••]
Herpes simplex virus, UL15 protein	F	R	Q	L	V	H	F	V	R	D	F	A	Q	L	L		
Adenovirus 12, open reading frame	D	F	E	V	V	T	F	L	K	D	V	L	P	E	F		
Pseudomonas, phosphomannomutase	D	R	L	L	M	L	F	A	K	D	V	V	S	R	N		
Human papillomavirus 7, L2 protein	I	G	G	R	V	H	F	F	K	D	I	S	P	I	A		
Human CD4+ clone, HLA-DR2-restricted																	
†Myelin basic protein (85–99)	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R		[35••]
Epstein–Barr virus, DNA polymerase	T	G	G	V	Y	H	F	V	K	K	H	V	H	E	S		
Influenza A, hemagglutinin	Y	R	N	L	V	W	F	I	K	K	N	T	R	Y	P		
Reovirus 3, sigma 2 protein	M	A	R	A	A	F	L	F	K	T	V	G	F	G	G		
Human CD4+ clone, HLA-DR2-restricted																	
†Myelin basic protein (85–99)	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R		
Epstein–Barr virus, DNA polymerase	T	G	G	V	Y	H	F	V	K	K	H	V	H	E	S		[35••]
Influenza A, hemagglutinin	Y	R	N	L	V	W	F	I	K	K	N	T	R	Y	P		
Herpes simplex, DNA polymerase	G	G	R	R	L	F	F	V	K	A	H	V	R	E	S		
Human CD4+ clone, HLA-DRB4•0101-restricted																	
†Pyruvate dehydrogenase complex-E2	G	D	L	L	A	E	I	E	T	D	K	A	T	I			[59••]
E. coli, pyruvate dehydrogenase complex-E2	E	Q	S	L	I	T	V	E	G	D	K	A	S	M			
Mouse CD8+, $H2D^b$-restricted																	
†Mycobacteria hsp60	S	A	L	Q	N	A	A	S	I	A							[58••]
Mouse hsp60	K	D	I	G	N	I	I	S	D	A							

*As measured by proliferation assays. †Natural ligand.

[43••]. One possible interpretation is that these transgenic T cells have altered the resting threshold so that the T cell does not 'react' to the endogenous antagonist Hb peptides, supporting the mechanism of T cell tuning. Other models support the possibility of peripheral T cell tuning to different concentrations or of temporal expression of a given ligand [47,48••].

Self peptides and autoimmunity

Because accumulating evidence suggests a single TCR may recognize analogous epitopes, it expands the possibility that autoimmunity may be induced by a mechanism initially described as molecular mimicry [49–53,54••]. Infection with a viral or bacterial pathogen will elicit an immune response against various antigenic epitopes, which may cross-react with self peptides that consequently become the target of an autoimmune response. This would imply that certain disease-associated alleles would be directly involved in the processing and efficient presentation of self peptides that are homologous to peptides derived from common pathogens. It is also possible that predisposition to autoimmunity may involve peptide-based positive selection of autoreactive T cells.

Studies by Tian and co-workers have demonstrated that glutamamic acid decarboxylase 65, a target for autoimmunity in nonobese diabetic mice [55,56], and coxsackievirus P2-C have homologous epitopes that are processed and presented by the class II molecules of the nonobese diabetic mouse [57] (Table 1; certain proteins are included in Table 1 for comparison only and will not be discussed in the text). Notably, neither of these epitopes could be presented by nine alternative class II alleles, providing a correlation between disease-susceptible strains and peptide presentation.

Heat shock proteins (hsps) are highly conserved from prokaryotes to eukaryotes and may provide a link between pathogenic proteins that are directly presented to the immune system and cross-reactive self molecules that are targets of autoimmunity. Zugel *et al.* [58••] have recently isolated a cytotoxic T lymphocyte line after immunizing mice with mycobacterial hsp60, and have identified cross-reactive ligands derived from the mouse hsp60 protein. The peptides that are recognized by the same clone show limited amino acid homology (SALQNAASIA and KDIGNIISDA for mycobacterial hsp60 and murine hsp60, respectively; Table 1).

Interesting studies have also been done by examining autoreactive T cells for ability to respond to cross-reactive epitopes from different pathogens [35••,59••,60]. Viral and bacterial peptides were able to induce proliferation of myelin basic protein specific human T cell lines (Table 1) [35••]. Similarly, human T cell clones from patients with primary biliary cirrhosis showed cross-reactivity to the homologous *Escherichia coli* peptide (Table 1) [59••]. It is important to note that many of these studies are performed with T cell clones, which have been maintained by stimulation with antigen *in vitro*. Therefore, the cross-reactivity to self ligands is measured at the effector cell stage, and these cross-reactive ligands may not normally be capable of activating an immune response *in vivo*. These studies provide important evidence of links between autoimmunity and infectious agents and a possible mechanism for the induction of autoimmune diseases.

Conclusions

T cell interactions are generally well defined and specific for a given ligand. Evidence also suggests, however, that considerable flexibility exists between TCR–ligand interactions during T cell development and effector T cell recognition. This may be an important mechanism to increase the diversity of the T cell repertoire; however, this flexibility must be limited in some way to avoid autoimmunity. TCR tuning during the lifespan of a T cell may alter the resting threshold of T cells, and modify the potential spectrum of activating ligands to maintain self tolerance. Therefore, two major mechanisms may exist that define the fine specificity of the T cell repertoire: clonal deletion of T cells with high-affinity for self ligands, and T cell tuning towards lower-affinity self ligands. Future studies may address the possibility of T cell tuning in other models [61,62] and help to identify potential molecules involved in tuning. Further studies may also explore potential relationships between selecting peptides, pathogen-derived peptides and self peptides that are the targets for an autoimmune response.

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1. European Journal of Immunology:

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Expression of HLA Class I Molecules and the Transporter Associated With Antigen Processing in Hepatocellular Carcinoma

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The expression of the HLA class I molecules on the cell surface was investigated in hepatocellular carcinoma (HCC) cell lines using complement-mediated cytotoxicity (CMC) and flow cytometric analysis. Although HLA-A antigens were detected by CMC in all cell lines tested, HLA-B and -C antigens were not detectable in six of seven HCC cell lines. These results were also confirmed by flow cytometric analysis focusing on HLA-Bw4 and Bw6 public antigens. Furthermore, complementary DNA (cDNA) from each cell line was tested for the expression of HLA-A, -B, -C and the transporter associated with antigen processing genes (TAP1 and TAP2). Two cell lines showed a reduced level of one or both of the TAP messenger RNAs (mRNAs), and one of these showed a reduction of HLA-B and -C gene expression as well, but the others had detectable mRNA levels. These results demonstrate that hepatocellular carcinoma cell lines tested in the current study lose or decrease the expression of HLA-B and -C alleles on the cell surface, even though mRNA encoding these alleles is present, suggesting that the loss of the HLA molecules might be caused by posttranscriptional events or failure to transport and load peptides necessary for HLA expression. The selective loss of HLA-B and -C, but not -A, molecules (which also excludes a β_2-microglobulin defect) is intriguing, and may be attributable to the ability of some of the HLA-A molecules to load signal peptides not requiring TAP transport, or to natural selection by HLA-B or -C locus-specific immune surveillance. (HEPATOLOGY 1996;23:1181-1188.)

It is commonly accepted that the expression of HLA class I molecules on the cell surface is pivotal for the recognition of tumor cells by cytotoxic T lymphocytes (CTL), because CTL recognize the endogenously processed antigenic peptides in the association with HLA molecules expressed on the cell surface.[1-4] In the case of tumor cells, the endogenous peptide of interest is probably from a self protein that has incurred a mutation or from an overexpressed wild-type protein. Extensive efforts have been applied for eluting and identifying these tumor antigens, and some tumor-specific antigens that can be recognized by class I-restricted CTL have been identified and characterized.[5] The recognition of a tumor cell by CTL is regulated by the interaction between T-cell receptor and the ternary complex of HLA, antigenic peptide, and β_2-microglobulin.[6] Therefore, the level of expression of HLA class I molecules on the tumor cell surface is a determining factor for the clearance of tumor by CTL. It has been reported that some tumor cells downregulate the expression of HLA class I molecules on the cell surface.[7-9] This phenomenon is thought to be one of the mechanisms for malignant tumor cells to escape immune surveillance.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Nevertheless, the expression of HLA locus-specific antigens in HCC has not been well studied. Therefore, the cell surface expression of HLA class I molecules was evaluated in seven HCC cell lines established from different individuals using complement-mediated cytotoxicity (CMC) and flow cytometric analysis. Many lines of evidence have demonstrated that the cytokine interferon gamma (IFN-γ) increases the expression of HLA class I mole-

Abbreviations: CTL, cytotoxic T lymphocyte; HCC, hepatocellular carcinoma; CMC, complement-mediated cytotoxicity; IFN-γ, interferon gamma; PCR, polymerase chain reaction; cDNA, complementary DNA; TAP, transporter associated with antigen processing; ER, endoplasmic reticulum; FCS, fetal calf serum; MAb, monoclonal antibody; mRNA, messenger RNA; NK, natural killer.

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cules on the cell surface. Thus, cells treated with IFN-γ were also examined for the expression of HLA to determine whether the HLA expression could be upregulated in these tumor cells. In addition, the expression of HLA-A–, B–, and C–specific genes was investigated by the polymerase chain reaction (PCR) technique using complementary DNA (cDNA) made from extracted RNA. Recently, the transporter associated with antigen processing (TAP) was shown to be critical for delivering the antigenic peptides from the cytosol into the lumen of the endoplasmic reticulum (ER), where the peptides are complexed and assembled with major histocompatibility complex (MHC) class I molecule.[10-13] The TAP complex is a heterodimer consisting of the products of the TAP1 and TAP2 genes.[10] Deletion of or mutation in the TAP genes results in a drastic reduction in the expression of HLA molecules on the cell surface.[14] Therefore, TAP expression and stable HLA allele expression on the cell surface are likely to be closely related. Consequently, the presence of TAP1 and TAP2 genes was also examined by the PCR.

TABLE 1. Nucleotide Sequence of the Primers

Locus	Primer Pair (5'→3')	Location
HLA-A	TGAACGAGGACCTGCGCTCT	Exon 3
	+CGAGCTCCGTGTCCTGGGTC	Exon 4
HLA-B	ACGGGCGCCTCCTCCGCGGGC	Exon 3
	+CGCCCTGAACGAGGACCTGA	Exon 3
	+AGGTTCTATCTCCTGCTGGT*	Exon 4
HLA-C	GACGCCGCGAGTCC(A/G)AGAGG	Exon 2
	+CACGTGTGTCTTTGGG(T/G)GT	Exon 4
TAP1	CCGCCTCACTGACTGGATTC	Exon 2
	+GCACGTGGCCCATGGTGTTGTTATAG	Exon 3
TAP2	GCCGAGCATGAAGTCTG	Exon 5
	+CCACGCTCTCCTGGTAGATC	Exon 6
Tubulin	TCCTTCAACACCTTCTTCAG	Exon 2
	+TGGCCTCATTGTCTACCATG	Exon 4

NOTE. For the detection of HLA-B alleles, two 5' primers were prepared to amplify all known B alleles (*). The expected base pair sizes for each locus are as follows: A locus, 295 bp; B locus, 405 bp or 350 bp; C locus, 453 bp; TAP1, 132 bp; TAP2, 218 bp; Tubulin, 484 bp.

MATERIALS AND METHODS

Cell Lines. HCC cell lines established from different patients with HCC were obtained from The Japanese Cancer Research Resources Bank.[15-17] Among the seven cell lines, three (huH-1, huH-2, and PLC/PRF/5) were derived from patients with hepatitis B virus infection and others were from patients without hepatitis B virus. HLE and Hep-3B were cultured in complete T-cell medium (1:1 mixture of RPMI 1640 and Eagle-Hank's amino acid medium) with 10% heat-inactivated fetal calf serum (FCS). HuH-7 was cultured in RPMI 1640 with 2% FCS and 30 nmol/L Na_2SeO_3. Other HCC cell lines were cultured in DM-160 medium (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan) with 10% FCS. A normal liver cell line, immortalized by transfecting with the plasmid containing SV40 T-Ag,[18] was maintained in RPMI 1640 with 5% FCS. All media contain 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5×10^{-5} mol/L 2-ME. Cells were split by treating with 0.05% trypsin-ethylenediaminetetraacetic acid (Life Technologies) every week, and half of the cells were returned to the flask for further culture. The T2 cell line that is deficient in TAP1 and TAP2 gene was provided by Dr. Peter Cresswell (Yale University, New Haven, CT).

HLA Phenotype Analysis by CMC. HLA typing was performed by CMC using the Amos Modified method. Briefly, 2 days before the HLA typing, cells were split by treating with 0.05% Trypsin-ethylenediaminetetraacetic acid for 3 minutes at 37°C. After being washed twice with RPMI 1640, 2×10^6 cells were resuspended in 10 mL of the appropriate medium and returned to 25-cm^2 tissue culture flasks. For the cells treated with IFN-γ, a solution of recombinant human IFN-γ (Boehringer Mannheim Biochemica) was added to the flask. After addition of 1 μL of a thoroughly mixed cell suspension adjusted to 1.0×10^6 cells/mL to each well of the typing trays containing different HLA-specific antibodies, the trays were incubated at room temperature for 50 minutes and washed with RPMI 1640 with 1% FCS, and allowed to settle for 15 minutes before they were flicked. Then, 5 μL of preabsorbed rabbit complement was added to the well, and the trays were incubated at room temperature for 60 minutes. After addition of 5 μL of Trypan containing ethylenediaminetetraacetic acid solution to each well, the trays were allowed to settle for 30 minutes at room temperature, then the trays were read on a fluorescent inverted microscope, and reaction scores were recorded.

Flow Cytometric Analysis. The treatment of the cells before the analysis was done the same way as described. Forty-eight hours after the last passage, 4×10^5 cells per well in flow microfluorometry medium (Hanks' balanced salt solution [without Ca, Mg, Phenol red], 0.2% bovine serum albumin, and 0.1% sodium azide) were added to 96-well U-bottom plates. After centrifugation and removal of the supernatant, the cells were incubated with or without the first appropriate antibody for 30 minutes at 4°C. After being washed twice with 100 μL of FMF medium, cells were further incubated for 30 minutes at 4°C with fluorescein isothiocyanate–conjugated secondary antibodies. The pellets were resuspended with FMF medium, and the fluorescence detection was performed with a FACScan (Becton Dickinson, San Jose, CA). To evaluate the difference in fluorescence intensity among the cells, all lines were analyzed using the same FL1 settings. Ten thousand events were acquired for each analysis.

Antibodies for Flow Cytometric Analysis. For detecting the HLA-B locus antigens, anti–HLA-Bw4 (monoclonal antibody [MAb], Biotest AG 6072 Dreieich, Germany) and anti–HLA-Bw6 (MAb, SFR8-B6 [rat immunoglobulin G2b] hybridoma culture supernatant)[19] were used. These two MAbs were provided by Dr. Francesco M. Marincola (National Cancer Institute, National Institutes of Health, Bethesda, MD). To detect HLA-A2, anti–HLA-A2 MAb (immunoglobulin G2b) was purified from the culture supernatant of hybridoma BB7.2[20] (American Type Culture Collection, Rockville, MD), using Gammabind-G sepharose (Pharmacia).

RNA Preparation and Analysis by PCR. Total RNA of each HCC cell line was prepared using the RNA STAT-60 isolation reagent (Tel-Test "B", Inc., Friendswood, TX). RNA (500 ng) was reverse transcribed and then amplified (30 cycles) using the *GeneAmp* RNA PCR Kit (reverse-transcription PCR; Perkin Elmer Cetus, Norwalk, CT). Five microliters of amplified product was run on 6% acrylamide gels and stained with ethidium bromide. Amplification of tubulin from cDNA of the various HCC cell lines was used to control for the amount of cDNA prepared from each cell line and loaded onto the gels.

TABLE 2. Analysis of HLA Class I Typing of Seven HCC Cell Lines and a Normal Liver Cell Line

Cell line	CMC Typing HLA Allele Expressed						Molecular Typing					
	A Locus		B Locus		C Locus		A Locus		B Locus		C Locus	
huH-1*	2	(−)	(−)	(−)	(−)	(−)	02	11	38	62(15)	01	07
Hep-G2*	2	(−)	(−)	(−)	(−)	(−)	02	24	51	35	04	
HuH-7*	11	(−)	(−)	(−)	(−)	(−)	11		54		01	
Hep-3B*	28	(−)	(−)	(−)	4	(−)	28(68)		14	35	04	08
huH-2*	2	(−)	(−)	(−)	(−)	(−)	02		35		0303	
HLE*	2	24	62(15)	(−)	(−)	(−)	02	24	15	15	01	0303
PLC/PRF/5*	3	(−)	(−)	(−)	(−)	(−)	03	33	5301	42	04	1701
NLCL*	28	(−)	58	(−)	6	(−)	28(68)		58		06	
huH-1†	2	11	38	62(15)	1	7						
Hep-G2†	2	24	51	(y)	4	(−)						
HuH-7†	11	(−)	54	(−)	1	(−)						
Hep-3B†	28	(−)	14	35	4	8						
huH-2†	2	(−)	35	(−)	3	(−)						
HLE†	2	24	62(15)	15	1	3						
PLC/PRF/5†	3	33	53	(y)	4	(−)						

NOTE. All cell lines were tested in the absence of IFN-γ (*), and all the HCC cell lines were tested after being treated with IFN-γ (500 U/mL) for 48 hours (†). (y) indicates that some reactivity was there, but it was not clearly identified. The molecular typing of the cell lines was performed using PCR-SSP.[45]

Primers for tubulin and all HLA loci used in PCR amplification are listed in Table 1.[21-23]

RESULTS

HLA Phenotype Analysis by CMC. HLA phenotypes of seven HCC cell lines were determined by the method of CMC. In all of the cell lines tested, HLA-A antigens were consistently detected by this method even in the absence of IFN-γ (Table 2). Only one HLA-A allele was detected in six of seven cell lines, the one exception being HLE, in which both HLA-A alleles were detected. Three cell lines (huH-1, huH-2, and Hep-G2) were shown to express HLA-A2 and other cell lines (HuH-7, Hep-3B, PLC/PRF/5) HLA-A11, 28, and 3. In contrast to these results for the HLA-A locus, no HLA-B and C antigens were detected in six of seven cell lines. Only one HLA-B allele was detected only in HLE. The HCC cell lines in which HLA-B antigens were not detected in the standard culture conditions were analyzed for HLA expression after being treated with 500 U/mL of IFN-γ for 48 hours, conditions known to enhance the expression of the HLA molecules.[24] Additional HLA-A alleles and new HLA-B and -C alleles were detected after IFN-γ treatment. Thus, in these cases, the genes were intact and could be expressed if induced with IFN-γ.

Flow Cytometric Analysis. Lack of HLA-B antigen expression by CMC led us to apply flow cytometry analysis to the problem. To test more precisely the level of HLA-B antigen expression on the HCC cell lines, cells were stained with anti-Bw4 and anti-Bw6 antibodies, then analyzed by flow cytometry (Fig. 1 and Table 3). Bw4 is a public epitope on some A locus alleles (A23, A24, A25, and A32) and some HLA-B alleles. Bw6 is an epitope on B-locus alleles that lack Bw4. However, neither Bw4 nor Bw6 antigens were detectable in five of seven HCC cell lines in the standard culture conditions. Two cell lines (huH-2 and HLE) were shown to express Bw6 antigen. The huH-2 line expressed a low level of Bw6, whereas HLE expressed a high level of this antigen, and the flow cytometry histogram indicated a homogeneously stained population of HLE cells. This result is very consistent with that obtained by CMC, because the HLA-B62 allele detected by CMC contains the Bw6 determinant. In contrast to the lack of expression of HLA-B antigens on most HCC cell lines in the absence of IFN-γ, a normal liver cell line expressed a detectable level of the Bw4 antigen even in the absence of IFN-γ (Fig. 1b and Table 3). Subsequently, HCC cell lines treated with IFN-γ were subjected to the same analysis. The expression of HLA-Bw4 or HLA-Bw6 antigens was enhanced by the treatment with IFN-γ in all seven HCC cell lines (Fig. 1A and Table 3). Although expression of both Bw4 and Bw6 was increased by IFN-γ in PLC/PRF/5 and Hep-G2, only the expression of Bw6 was enhanced in other HCC cell lines. The expression of the Bw4 and Bw6 in Hep-G2 was less than that of other cell lines. Furthermore, the three cell lines shown to possess HLA-A2 by CMC were stained with anti–HLA-A2 specific antibody (BB7.2) and analyzed by flow cytometry. The expression of the HLA-A2 molecule by these cell lines in the standard culture conditions was confirmed and was shown to be upregulated also by IFN-γ.

One mechanism by which tumor cells can lose HLA expression is a loss of the TAP transporter genes necessary for loading peptide onto major histocompatibility complex class I molecule in the ER.[25,26] If this were the case, however, then IFN-γ would not be expected to restore the response. To examine the effect of IFN-γ in this situation, we analyzed the T2 cell line, in which TAP1 and TAP2 genes have been deleted, for the pres-

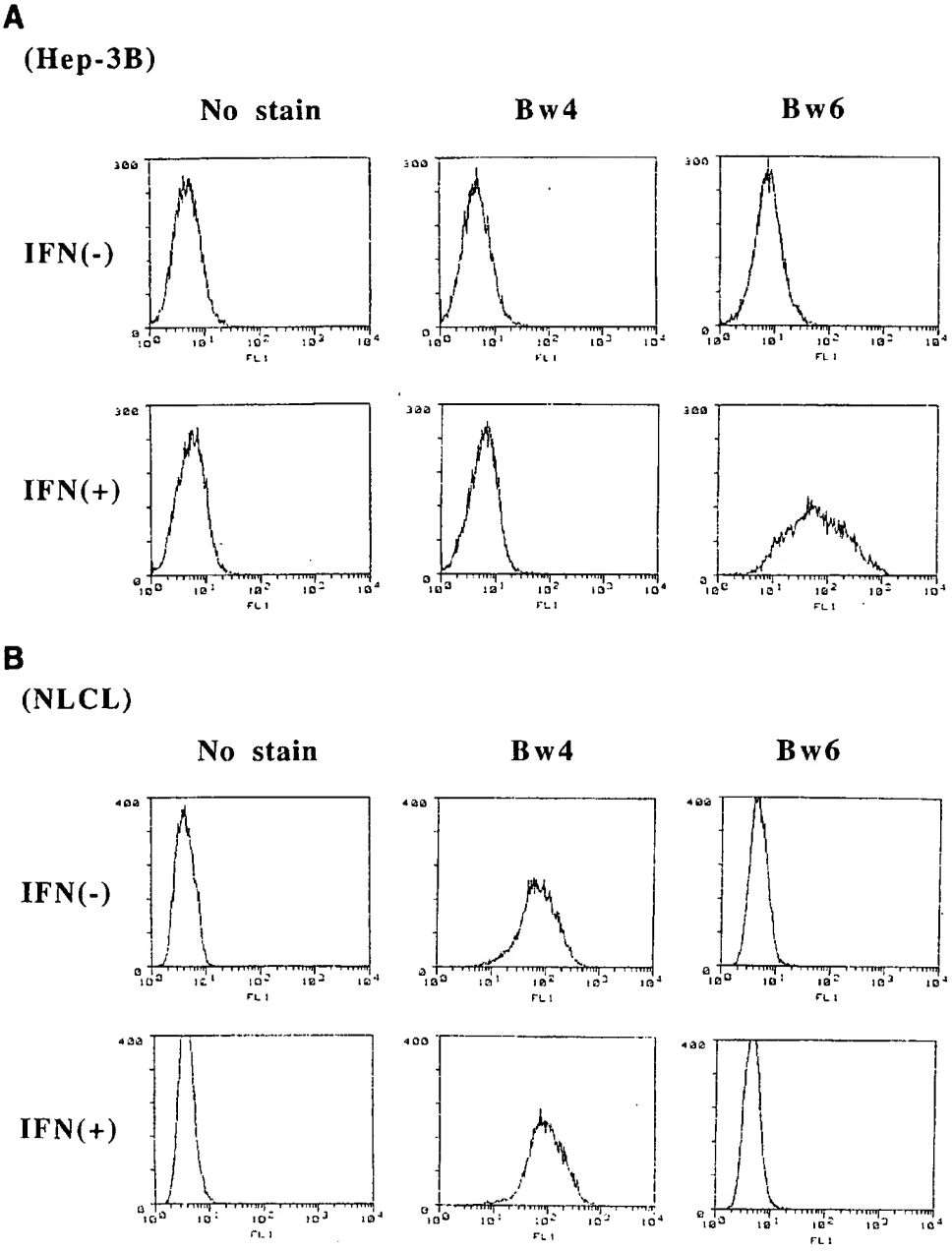

FIG. 1. (A) Fluorescence histograms of HLA-Bw4, Bw6, and A2 expression on one of the HCC cell lines (Hep-3B). Cells (4×10^5) were stained by these antibodies in the absence and presence of IFN-γ. Each histogram shows the number of stained cells and fluorescence intensity. (B) Fluorescence histograms of HLA-Bw4, Bw6, and A2 expression on a normal liver cell line (NLCL).

ence of HLA-B locus–specific antigens and the HLA-A2 antigen on the cell surface. HLA-Bw4 and HLA-A2 antigens on the cell surface of this cell line were detectable in the standard culture conditions, potentially because of the presence of empty class I molecules, exogeneously loaded peptides, or signal peptides transported without TAP.[27] However, expression of these antigens was not enhanced by IFN-γ treatment (Table 3).

Analysis of the Expression of the HLA and TAP Genes. To determine whether the lack of expression of the HLA antigens on the cell surface was regulated at the level of transcription, the cells were analyzed for HLA-A, -B, and -C messenger RNA (mRNA). cDNA made from RNA extracted from each cell line was assessed by PCR analysis using primers specific for each of the HLA-A, -B, and -C locus genes (Fig. 2 and Table 4). The expression of TAP1 and TAP2 genes was also examined, because the TAP molecule is critical for translocating antigenic peptides into the ER, a requirement for expression of the HLA class I molecules (Fig. 3 and Table 4). The results showed that HuH-7 had diminished expression of the TAP1 gene and the genes encoding HLA-B and -C molecules (Fig. 2 and Table 4).

TABLE 3. Flow Cytometric Analysis of Bw4, Bw6, and A2 Antigen Expression on HCC Cell Lines and a Normal Liver Cell Line

Cell Line	Δ Mean Fluorescence		
	BW4	BW6	A2
huH-1 [IFN(−)]	−0.19	3.19	32.11
huH-1 [IFN(+)]	52.91	114.05	98.62
Hep-G2 [IFN(−)]	4.17	1.29	13.85
Hep-G2 [IFN(+)]	26.03	16.91	32.31
HuH-7 [IFN(−)]	3.27	9.11	NT
HuH-7 [IFN(+)]	8.00	314.71	NT
Hep-3B [IFN(−)]	0.12	3.59	NT
Hep-3B [IFN(+)]	0.58	121.42	NT
huH-2 [IFN(−)]	−0.33	30.53	30.21
huH-2 [IFN(+)]	−0.07	170.39	100.08
HLE [IFN(−)]	0.21	230.63	NT
HLE [IFN(+)]	5.09	507.59	NT
PLC/PRF/5 [IFN(−)]	2.88	4.18	NT
PLC/PRF/5 [IFN(+)]	54.32	56.95	NT
NLCL [IFN(−)]	92.48	0.59	NT
NLCL [IFN(+)]	120.89	0.73	NT
T2 [IFN(−)]	22.52	4.79	16.5
T2 [IFN(+)]	19.8	4.92	14.96

NOTE. Each cell line was stained with anti-Bw4, Bw6, and A2 antibodies and analyzed by flow cytometry. Each number shows the Δ mean fluorescence of each sample.

Abbreviation: NT, not tested.

Also, Hep-3B was shown to have very little expression of both the TAP1 and TAP2 genes. However, the consistent presence of bands in single-strand conformational polymorphism analysis of TAP2 cDNA indicated that there was certainly some TAP2 mRNA present in Hep-3B (data not shown).[21] All other HCC cell lines were shown to express both HLA and TAP genes. Thus, low levels of HLA-B and C expression at the surface were not due to lack of HLA or TAP gene transcription in most cases. Posttranscriptional effects on HLA protein synthesis, peptide loading or transport, or on TAP protein production or function could account for the loss of surface expression.

DISCUSSION

The expression of HLA molecules on the cell surface is pivotal for recognition by T cells. In particular, the level of expression of the HLA class I molecules on tumor cells affects the recognition by $CD8^+$ cytotoxic T cells. It has been reported that the loss of expression of HLA class I molecules is one of the phenomena often observed in tumor cells.[7-9] Although it is speculated that this phenomenon is one of the mechanisms for tumor cells to escape recognition by T cells and immune surveillance, the underlying molecular mechanism is not well understood. The loss or downregulation of expression of HLA molecules has been reported in different types of tumors, including melanoma,[24,28] colorectal tumor,[29,30] squamous cell carcinoma of the uterine cervix[31] and of larynx,[32] and familial adenomatous polyposis.[33] In HCC, very few studies have examined HLA protein on the cell surface.[34] To investigate the expression of the HLA antigens, seven HCC cell lines were analyzed by CMC. In six of seven cell lines, HLA-B and -C alleles were not detectable, whereas HLA-A alleles were consistently detected in the absence of IFN-γ. Inability to detect HLA-B and -C molecules by the technique of CMC does not necessarily mean that HLA-B and -C locus antigens are not expressed on the cell surface. To confirm the results obtained by CMC, the more sensitive flow cytometry analysis was performed using antibodies to the public specificities Bw4 and Bw6.[24] Every HLA-B allele possesses one of these epitopes, with Bw6 present on two thirds and Bw4 on the remaining one third of HLA-B alleles.[35,36] Bw6 is found on only HLA-B molecules, but Bw4 is also found on some HLA-A alleles, including HLA-A 23, 24, 25, and 32. Neither Bw4 nor Bw6 antigens were detected in five HCC cell lines, but Bw4 antigen was clearly detectable on the control normal hepatocyte cell line in the absence of IFN-γ. However, the expression of Bw4 or

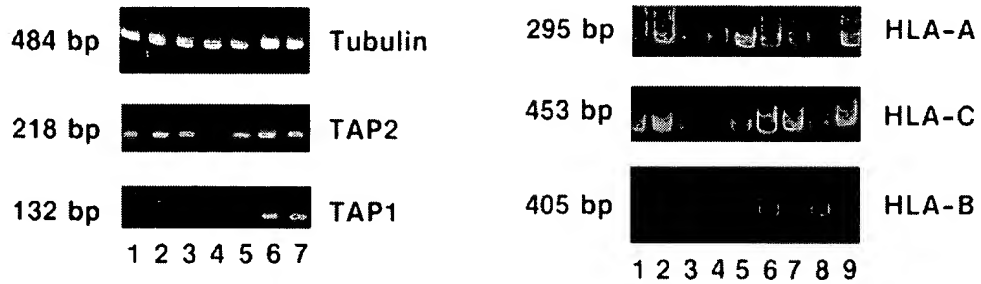

FIG. 2. Analysis of HLA and TAP gene expression of HCC cell lines. Total RNA of each HCC line was reverse transcribed and amplified using the primers for the detection of HLA-A, B, C, TAP1, and TAP2 sequences. Amplified product was run on acrylamide gels and stained with ethidium bromide. Each numbered lane shows the result from seven HCC cell lines, and two control B-lymphoblastoid cell lines: huH-1 (lane 1), Hep-G2 (lane 2), HuH-7 (lane 3), Hep-3B (lane 4), huH-2 (lane 5), HLE (lane 6), PLC/PRF/5 (lane 7), B-lymphoblastoid cell lines (lanes 8 and 9).

TABLE 4. Analysis of HLA and TAP Gene Expression by PCR

Cell Line	TAP1	TAP2	HLA-A	HLA-B	HLA-C
huH-1	+ +	+ + +	+ + +	+ + +	+ + +
Hep-G2	+ +	+ + +	+ + +	+ + +	+ + +
HuH-7	± ±	+ + +	+ + ±	— — ±	± ± —
Hep-3B	— ±	— ± ±	+ + ±	+ + —	+ ± +
huH-2	± +	+ + +	+ + +	+ + +	+ + +
HLE	+ +	+ + +	+ + +	+ + +	+ + +
PLC/PRF/5	+ +	+ + +	+ + +	+ + +	+ + +

NOTE. RNA extracted from each HCC cell line was transcribed and amplified using primers listed in Table 1. Amplified product was run on acrylamide gels and stained with ethidium bromide. The results of two or three individual experiments are shown. +, amplified band is clearly visible; ±, visible but light; —, not visible.

Bw6 antigens in HCC cell lines was enhanced by treatment with IFN-γ. These results indicate that the genes and expression pathways for these HLA-B antigens are intact, but the expression of these antigens is prevented by some regulatory mechanism.

This down-regulation of class I expression might be related to the development of malignancy or escape of tumors from immune surveillance by $CD8^+$ CTL that recognize tumor antigens presented by class I HLA molecules. In contrast, it is believed that natural killer (NK) cells do not require HLA molecules as restriction elements, but rather favor target cells lacking surface HLA molecules. However, very recently, HLA class I molecules, especially but not exclusively HLA-B, have been shown to affect NK cell reactivity, and the existence of NK cell receptors involved in the recognition of HLA-B molecules has been demonstrated.[37-39] In contrast to conventional CTL, however, recognition of the appropriate HLA molecule inhibits NK cell function. Thus, the down-regulation of expression of HLA-B antigens should enhance recognition by NK cells and could not account for the escape from NK cell surveillance.

It is also intriguing that only one allele of HLA-A locus is expressed on the cell surface of the HCC cell lines reported here. Because most of the HCC lines tested are capable of expressing additional A alleles after IFN-γ treatment, expression of a single HLA-A molecule in untreated cells cannot be caused by homozygosity at the HLA-A locus. Therefore, it is possible to speculate that the nonexpressed HLA-A allele might be downregulated by the same mechanism as are the HLA-B and -C alleles. Successful expression of the other A allele may result from the ability to circumvent the defects that restrict expression of the undetected alleles, e.g., rescue by presentation of signal sequences peptides in the presence of a mutated TAP protein.[27] One could hypothesize that down-regulation of B and C alleles and one A allele would be sufficient to allow escape from $CD8^+$ CTL, and the other A allele might serve to suppress recognition by NK cells, thus resulting in tumor outgrowth.

Without a sample of fresh tumor from which each tumor cell line was developed, it is impossible to exclude the possibility that this down-regulation of expression of HLA molecules on the cell surface might be caused by the long-term cell culture of HCC cell lines. As a control for the effects of long-term culture on hepatocytes, we used a normal liver cell line, (NLCL), which has been maintained long term in culture.[18] HLA-B and -C alleles (HLA-B58, Cw6) were detected in this cell line by CMC, and HLA-Bw4 antigen was also clearly detected by flow-cytometric analysis even in the absence of IFN-γ. These results suggest that long-term cell culture does not necessarily lead to down-regulation of HLA expression by hepatocytes and thus should not be the cause of down-regulation of HLA molecules in the HCC cell lines. In the absence of immune selective pressure *in vitro*, a tumor line could spontaneously lose HLA expression by chance, but it is unlikely that six of seven HCC cell lines would selectively lose HLA-B expression. This suggests that the loss occurred in the original tumors *in vivo*.

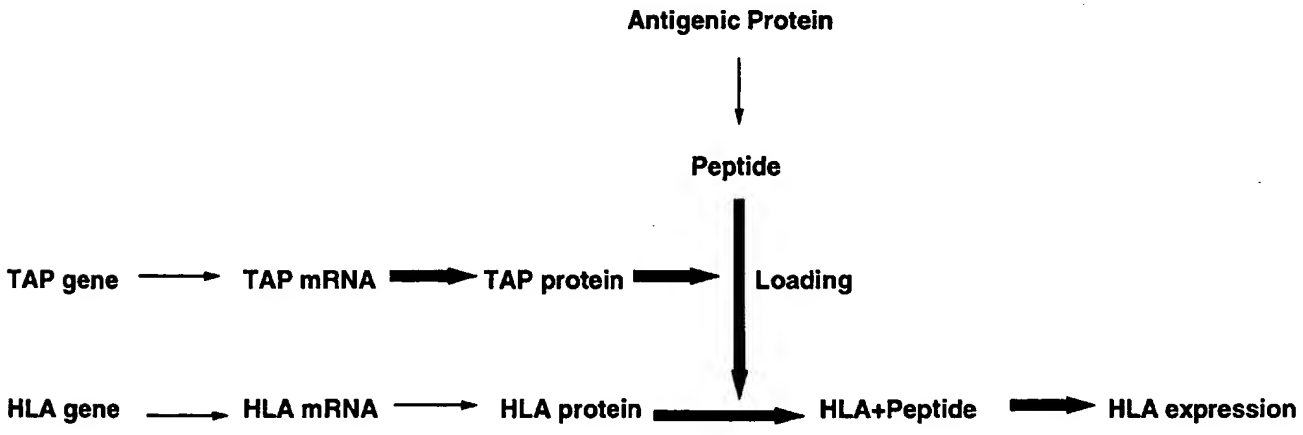

FIG. 3. Schematic diagram of steps in HLA class I expression. Large arrows show predictable locations of the causes potentially altering HLA expression in HCC lines studied.

To elucidate the underlying mechanism for the loss of HLA protein expression, we examined the HLA gene expression in HCC cell lines. Because a number of mechanisms are thought to induce loss of HLA expression in many tumors,[40] down-regulation of the HLA molecules on HCC cell lines may be caused by suppression of transcription or genomic loss of the genes encoding the HLA heavy chains, β_2-microglobulin, or TAP1 and TAP2 transporters. Although some reports have shown that a defect of β_2-microglobulin gene expression leads to lack of HLA class I protein expression in melanoma,[41,42] β_2-microglobulin must be expressed in HCC cell lines studied, because it is required for cell surface expression of HLA-A molecules. To test the possibility that the HLA-B molecules may be more dependent on TAP function than are some of the HLA-A molecules, the presence of TAP mRNA was examined, as was HLA heavy-chain mRNA. One HCC cell line (HuH-7) had low expression of HLA-B and C and TAP1 mRNA, and another line (Hep-3B) had diminished expression of both TAP genes. All other HCC cell lines expressed high levels of both HLA and TAP mRNA. It should be noted, however, that the reverse-transcription PCR method used here gives only a rough estimate of mRNA levels and is semiquantitative, because amplification by PCR is nonlinear. Previous reports have described a correlation between HLA loss and TAP gene defects in cervical carcinoma[26] and colorectal cancer.[25] However, in the HCC cell lines examined, neither genomic deletion nor complete lack of expression of these genes is the cause for the down-regulation of the expression of HLA molecules on the cell surface, although decreased TAP expression may contribute to the lack of HLA-B and -C allele expression in HuH-7 and Hep-3B. The fact that HLA-B molecule expression is enhanced in some of the lines by IFN-γ, which up-regulates transcription of both HLA heavy chain and TAP genes, suggests that overexpression of mRNA for one or both of these can overcome the defect in expression of HLA molecules on the cell surface. In Hep-3B, where TAP mRNA expression is relatively low (Fig. 2 and Table 4), reduced peptide loading may explain the poor surface expression of HLA-B molecules. It is possible that mutations in the TAP genes of the cell lines expressing high levels of class I and TAP mRNA could explain the deficient class I expression on the cell surface.

The expression of HLA-B alleles on the T2 cell line was examined by flow-cytometric analysis. The T2 cell line was derived from a somatic cell hybrid of T-B lymphoblastoid cell lines and is deficient in genomic DNA encoding TAP1 and TAP2 transporter subunits. Only HLA-A2 and a very low level of HLA-B5, a molecule containing the Bw4 public epitope, are expressed on the cell surface.[43,44] Expression of HLA-A2 on the surface of T2 cells can occur in the absence of TAP because HLA-A2 binds signal peptides that are translocated into the ER without the assistance of TAP.[27] Expression of HLA-A2 and Bw4 by T2 cells was not enhanced by the treatment with IFN-γ. Thus, the increased expression of HLA-B alleles after IFN-γ treatment in all of the HCC cell lines tested may result from increased expression of TAP after such treatment.

In conclusion, selective loss of expression of HLA-B and -C locus alleles on the cell surface was shown in six of seven HCC cell lines tested, even though HLA-A molecules were consistently expressed. The presence of HLA heavy chain and TAP genes and mRNA expression was also shown, suggesting that the loss or decreased expression of HLA molecules might be caused by posttranscriptional events or failure to transport and load peptides necessary for HLA expression (Fig. 3).

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IMMUNOLOGY TODAY

How does TAP associate with MHC class I molecules?

Tim Elliott

Major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum (ER) are in physical association with a number of cofactors, including the transporter associated with antigen processing (TAP) and a calcium-binding chaperone. Here, Tim Elliott suggests a molecular model for the way in which these cofactors could regulate the assembly and release of newly synthesized MHC class I molecules.

Most nucleated cells express major histocompatibility complex (MHC) class I molecules, which present antigenic peptides to cytotoxic T lymphocytes (CTLs). MHC class I molecules are transmembrane glycoproteins comprising a 45 kDa heavy chain (HC) with three extracellular domains (α_1, α_2 and α_3), noncovalently associated with β_2-microglobulin (β_2-m). These subunits are assembled in the endoplasmic reticulum (ER) in association with peptide epitopes that are derived from the cytosol and delivered to the ER by the transporter associated with antigen processing (TAP). Binding of a high-affinity peptide stabilizes the HC–β_2-m complex and signals its release from the ER. In this way, MHC class I molecules are continually sampling the internal composition of the cells in which they are expressed, before finally displaying their findings at the cell surface to circulating CTLs.

The macromolecular 'loading complex'

TAP molecules could contribute to peptide loading simply by raising the concentration of free peptide in the lumen of the ER. However, the discovery in 1994 that MHC class I molecules in the ER can be co-precipitated with TAP by anti-TAP antibodies[1–3] raised the possibility that TAP might play an active role in the assembly of class I molecules. It is now known that HC assembles with β_2-m soon after its synthesis and, within ten minutes, can be found in association with TAP (Ref. 1). TAP binds to these 'empty', peptide-receptive, HC–β_2-m heterodimers but not to stable, peptide-loaded molecules[1,2]. In fact, the ATP-dependent delivery of peptides to TAP–class I complexes results in the release of class I from TAP (Refs 1, 2). This release is peptide specific and is dependent on the MHC class I allele involved. The rate of release from TAP also mirrors the release of peptide-loaded class I molecules into the secretory pathway. Thus, it seems that, for most of their time in the early secretory compartment, class I molecules are associated with TAP.

Recently, Sadasivan *et al.*[4] have shown that, in human B-cell lines, TAP$^-$ class I complexes are found associated with two additional molecules: the ER-resident calcium-binding chaperone calreticulin, and a novel 48 kDa glycoprotein called tapasin (first seen in immunoprecipitates of TAP from digitonin lysates[2]). Similarly, in mouse cells, Suh *et al.*[5] have demonstrated a multi-component complex in which the majority of TAP-associated mouse class I molecules are simultaneously associated with another ER-resident calcium-binding chaperone, calnexin. In both cases, over half of the ER-resident pool of class I molecules is simultaneously associated both with TAP and with a calcium-dependent chaperone, while the rest is associated with the chaperone alone. Since TAP does not bind to calreticulin in the absence of class I, it is likely that the chaperone is required for formation of the ternary complex with TAP, whereas it is the class I molecule and not the chaperone that binds directly to TAP. The biogenesis of this chaperone–class I complex is therefore of crucial importance for initiating and maintaining an interaction with TAP.

In mouse and human cells, HC associates with calnexin soon after its synthesis, via interactions both with the immature glycan and with residues in the transmembrane domain of HC. In mouse cells, β_2-m binds to HC while the latter is bound to calnexin; whereas, in human cells, it appears that β_2-m binding displaces calnexin, and the resulting HC–β_2-m heterodimer binds instead to the homologous ER chaperone calreticulin (Fig. 1). It is not yet clear why there should be a difference in the choice of chaperone between the two species. Although calnexin is not essential for the assembly of mouse or human HC–β_2-m heterodimers, it may serve to increase the efficiency of assembly either directly or simply by retaining empty class I molecules in the early secretory compartment.

Sadasivan *et al.*[4] have shown that tapasin is essential for the interaction between class I and TAP since its absence in the recently described cell line LBL721.220 (Refs 6, 7) results in the inability of some MHC class I alleles to associate with TAP. As a consequence, these alleles remain in the ER as unstable 'empty' molecules. No murine homologue of tapasin has been reported to date. The gene encoding tapasin is most probably located in the region of chromosome 6 between 6p11 and 6pter (Ref. 6); this region also includes the MHC. Tapasin probably binds to the calreticulin–class I complex via a direct interaction with class I (as shown in Fig. 1), since anti-calreticulin antibodies co-precipitate TAP only in cells that express HC and β_2-m. Class I molecules could associate with TAP via this tapasin bridge alone, or might interact directly with both tapasin and TAP. Since the expression of different class I alleles appears to depend on the presence of tapasin to different degrees, the relative contribution of TAP and tapasin to a 'productive' interaction may vary between alleles[7]. Indeed, the very requirement for a physical interaction between class I and TAP for antigen presentation may vary between alleles[8]. Whether tapasin simply acts as a tether for

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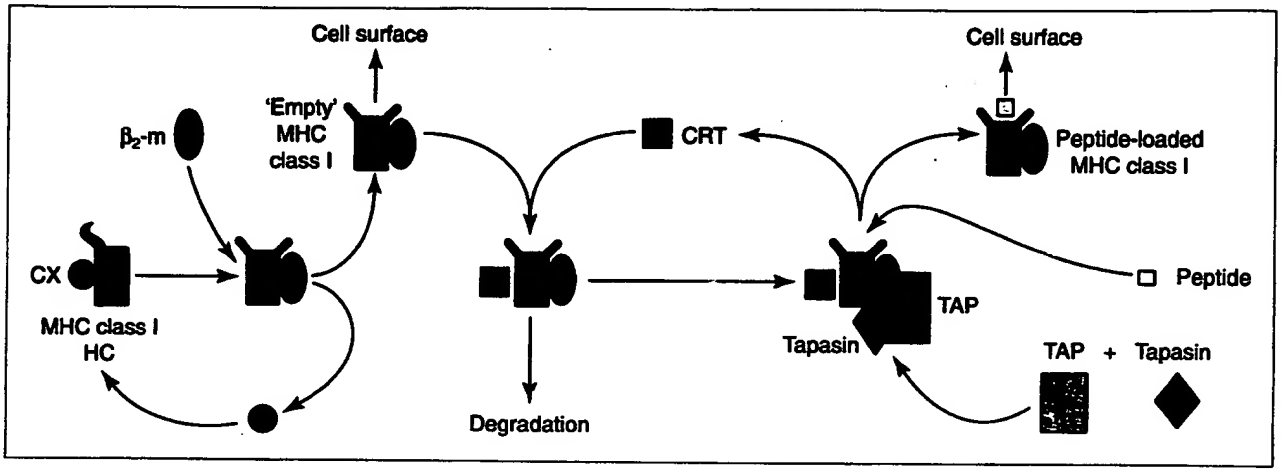

Fig. 1. *The role of ER-resident cofactors in the assembly of MHC class I molecules. In this hypothetical scheme, newly synthesized MHC class I HCs associate with the ER-resident cofactor CX until they bind to β_2-m. This displaces CX, and the peptide-receptive class I molecule is released into the secretory pathway where it is free to travel to the cell surface. Binding of this complex to a second cofactor, perhaps CRT, effects its retention in the ER, where it can bind to the TAP–tapasin complex. When TAP is absent, or when the peptide supply is limiting, the class I molecule is degraded in the ER. Upon peptide binding, the loaded class I molecule is released from the loading complex (comprising CRT, TAP and tapasin), and is transported to the cell surface. In the murine system, the later stages of MHC class I chaperoning by CRT may instead be achieved by CX, which is not displaced by β_2-m. Abbreviations: β_2-m, β_2-microglobulin; CRT, calreticulin; CX, calnexin; ER, endoplasmic reticulum; HC, heavy chain; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing.*

class I or whether it fulfils a more active role in peptide loading of class I molecules is also not known. For example, tapasin could itself bind to peptides delivered to the ER by TAP and pass them on to class I molecules. This is reminiscent of the role played by the oligopeptide-binding protein (OppA) of bacteria, which binds to peptides of 2–5 amino acid residues in the periplasm and delivers them to the Opp transmembrane transporter complex (a homologue of TAP)[9].

In the ER, therefore, newly synthesized class I molecules form part of a macromolecular assemblage: it is possible that the class I HC could be in simultaneous association not only with β_2-m and peptide, but also with a calcium-binding chaperone, tapasin and TAP. It appears that each of these contacts is essential for maintaining the overall stability of the complex.

Maintaining a stable loading complex

Studies on the interaction between TAP and several chimaeric mouse class I molecules have shown that the extracellular domains of classical MHC class I molecules are sufficient for their peptide-regulated interaction with TAP (Ref. 5). The region of the α_3 domain at residue E222 is important in maintaining the interaction, since a point mutation (E to K) at this position ablates binding without affecting the ability of the empty receptor to assemble; interestingly, this region forms part of the binding site for CD8. Both the region of the α_3 domain at residue 227 and β_2-m have also been implicated in the association between TAP and class I (Ref. 10). However, it is not clear whether the inability of these mutant molecules to associate with TAP impairs their ability to assemble with β_2-m or to present endogenous antigens.

More recently, residues in the α_2 domain have also been shown to be important in controlling the interaction between TAP and MHC class I. Indeed, a T to K mutation at position 134 (T134K) of HLA-A2.1 disrupts its interaction with TAP (Refs 11, 12). This residue lies within a highly conserved, solvent-exposed region of the α_2 domain. What is special about this mutation is that it inhibits the ability of HLA-A2.1 to present endogenous (TAP-supplied) peptide antigens, therefore implying that this region of the molecule has some role in maintaining a physiologically relevant interaction with TAP.

In addition to failing to interact with TAP, mutant T134K molecules are also rapidly transported to the cell surface as empty receptors and escape degradation in the ER: normally, class I molecules that are only partially assembled, due to a limited supply of peptides, are retained and degraded in the ER. This leads to the conclusion that the T134K mutation disrupts an interaction with an accessory molecule that is responsible both for the retention in the ER of class I molecules awaiting loading and for the sorting of unloaded molecules to the degradative pathway when these are in excess[11]. One strong candidate for this accessory molecule is tapasin (although it should be pointed out that wild-type HLA-A2.1 is expressed normally in the tapasin-negative cell line LBL721.220). Other candidates are the ER-resident chaperones calnexin and calreticulin, which are known to have a function in ER retention and quality control of nascent proteins, and now appear to be involved in the late stages of class I assembly. It was recently shown that the T134K mutant does not associate with calreticulin, suggesting that calreticulin could perform an essential function in the quality control of newly assembled MHC class I molecules (T. Elliott, unpublished).

One of the most important observations concerning the association between MHC class I molecules and TAP is that, upon peptide binding by class I and the completion of class I assembly, the macromolecular complex disperses. Recent experiments suggest that peptide binding by class I molecules initiates a step-wise disassembly of the complex, starting with chaperone–class I release from

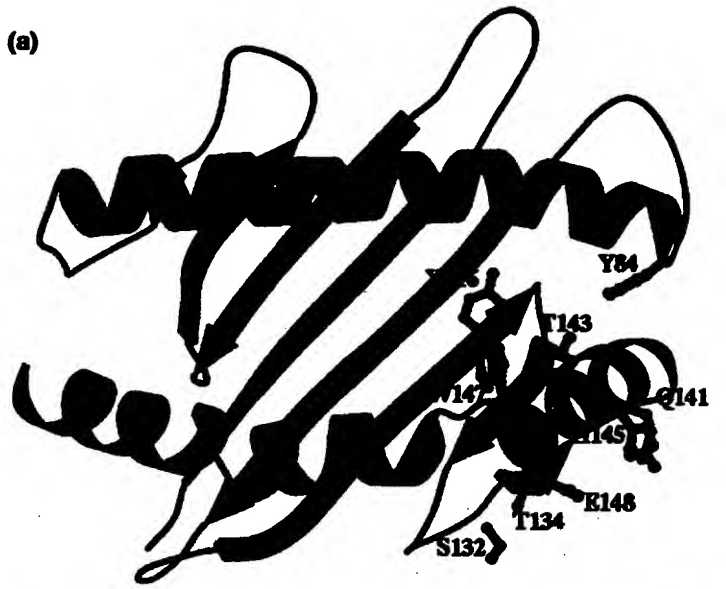

Fig. 2. *(a) The short α_2 helix may contain side-chains that are important for binding to cofactors, as well as side-chains that are important for binding to peptides. This ribbon diagram of the HLA-A2.1 α_1 and α_2 domains shows that the 'inner' face of the short α_2 helix contains residues W147 and T143 which, together with Y84, form a hydrogen-bonding network with the peptide C-terminal end that is essential for stable peptide binding. In addition, the polymorphic residue at 116 (which is Y in HLA-A2.1) contributes to the peptide-binding specificity. Several polar or charged residues lie on the 'outer' face of the short α_2 helix: Q141, H145 and E148, together with residues on the adjacent β strand such as S132 and T134, could form part of a binding site for TAP, or other components of the macromolecular 'loading complex'. (b) The short α_2 helix of MHC class I molecules is mobile. The figure shows the overlaid backbone structures of five complexes of MHC class I with peptide: H-2D^b with ASNENMETM, green; H-2M3^a with N-formyl-MYFINILTL, cyan; H-2K^b with RGYVYQGL, magenta; H2-K^b with FAPGNYPAL, pink; HLA-B35 with VPLRPMTY, red; HLA-B53 with TPYDINQML, orange; HLA-A2.1 with a mixture of peptides, dark green; HLA-B53 with KPIVQYDNF, pale green. For a general review of MHC class I structure, see Ref. 18. The fact that the relative position of the short α_2 helix differs for different alleles, or when different peptides are bound to the same allele, suggests that this region of the molecule may be relatively mobile. The position of the short α_2 helices describes a single body rotation around an axis that runs through residues A136 and A153, which act as pivots. For abbreviations, see Fig. 1 legend.*

TAP–tapasin, followed by the release of class I from the calcium-binding chaperone[4,5]. What triggers this disassembly? One possibility is that a conformational change in the TAP heterodimer (which accompanies peptide transport), driven by the hydrolysis of ATP, is responsible. Although such conformational changes have been observed for other members of the ATP-binding cassette (ABC) transporter superfamily (of which TAP is a member), the fact that the transport by TAP of peptides that do not bind to class I does not signal the release of class I, makes this possibility less likely. Another possibility is that a peptide-induced conformational change in the class I molecule itself (similar to that postulated for the intracellular stabilization of peptide-loaded MHC class II molecules) triggers its dissociation from the complex. Such a conformational change in HCs is seen in the absence of β_2-m (Ref. 13) and has been implicated when peptide binds to empty HC–β_2-m heterodimers[14,15].

Peptide-regulated release of class I from the ER

The peptide-binding groove of the class I molecule is defined at one end by a deep pocket. This so-called F-pocket accommodates the C-terminal end and the C-terminal side-chain of the peptide ligand, and its contacts with the bound peptide contribute significantly to the overall binding strength.

The specificity of peptide binding is largely controlled by the polymorphic residue 116, which lies at the bottom of the F-pocket. Three hydrogen bonds are made between conserved residues lining the F-pocket and the peptide–ligand backbone[16]. Thus, T143 and Y84 bonds with the peptide carboxylate, while W147 bonds with the penultimate carbonyl oxygen of the peptide ligand. Residues T143 and W147 lie on the short α_2 helix (138–149) of the α_2 domain, which forms one side of the F-pocket, and point into the groove (Fig. 2a). This region of the class I molecule is particularly mobile, and appears to differ in its relative position depending on the class I allele and the bound peptide ligand (Fig. 2b; Ref. 17). The most dramatic displacement is observed in HLA-B3501 (Ref. 17), where this region is displaced by 1.5Å relative to other class I molecules. In HLA-A0201, its position is shifted by approximately 1Å depending on which peptide is bound in the groove.

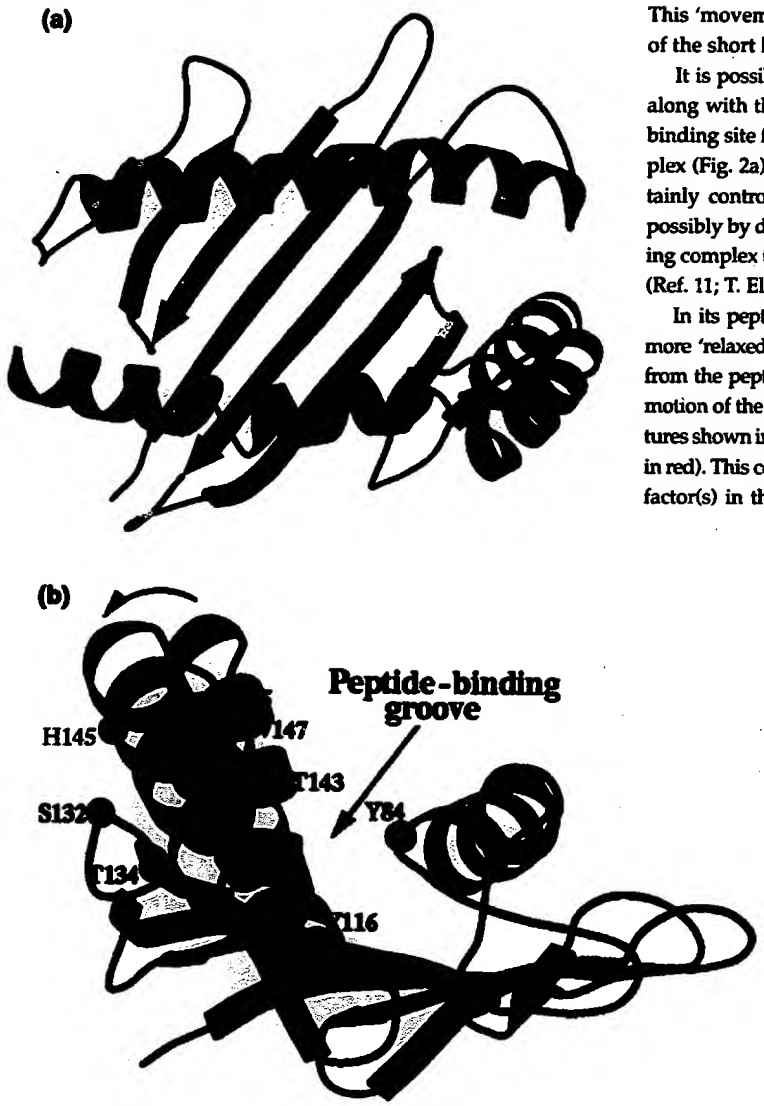

Fig. 3. *Peptide-receptive (red) and peptide-bound (green) MHC class I molecules may have different conformations dependent on the position of the short α_2 helix. Two views are shown: (a) looking down on the α_1 and α_2 domains; and (b) looking along the peptide-binding groove from the right-hand (F-pocket) end. The peptide-receptive conformation was generated by rotating the short α_2 helix through 45° around an axis that runs through A136 and A153. This leads to a displacement of the Cα atoms of H145 by 5.6Å and the Cβ atoms by 6.5Å. The main-chain torsion angles were kept in the α-helical conformation and the limit of rotation was determined by the side-chain of K144. Exposure of hydrophobic residues V152, I124 and Y123 was minimized – only W147 becoming considerably more exposed in the peptide-receptive conformation. The peptide-receptive conformation could have a high affinity for TAP (or other components of the 'loading complex') mediated in part via solvent-exposed residues on the outer face of the short α_2 helix (e.g. H145 in HLA-A2.1) and the adjacent β strand (e.g. S132 and T134). On peptide binding, the α_2 helix may be forced to move as the complement of specific and nonspecific interactions between MHC class I (involving W147, T143, Y116 and Y84) and peptide are formed. This might result in an MHC class I conformation that has a low affinity for TAP. For abbreviations, see Fig. 1 legend.*

This 'movement' is apparently achieved by a single body rotation of the short helix.

It is possible that the solvent-exposed side of the short α_2 helix, along with the conserved loop from 128–138, could form part of a binding site for one or more of the components of the loading complex (Fig. 2a). This region (132–134) of the class I molecule can certainly control the extent and timing of its interaction with TAP, possibly by disrupting an interaction with a component of the loading complex that is responsible for ER retention, such as calreticulin (Ref. 11; T. Elliott, unpublished).

In its peptide-receptive form, the F-pocket of class I may be in a more 'relaxed' conformation, with the short α_2 helix displaced away from the peptide-binding groove. Indeed, it is possible to extend the motion of the short α_2 helix along the same arc described by the structures shown in Fig. 2b to a limit imposed by steric factors (Fig. 3, shown in red). This conformation could create a high-affinity binding site for factor(s) in the loading complex, comprising residues on the outer face of the helix and the loop between 128–138. When the C-terminal end of an appropriate peptide ligand is introduced into this relaxed F-pocket, it may only be able to make the full complement of hydrogen bonds if the short α_2 helix is displaced inwards, towards the groove (Fig. 3, shown in green). This could disrupt the high-affinity cofactor-binding site and cause the dissociation of the fully assembled class I molecule from the loading complex. It is interesting to note that the degree to which TAP can be found in association with different class I alleles in digitonin lysates is dictated by the nature of the polymorphic side-chain at position 116 in the F-pocket[8]. Although it is not clear exactly how this residue affects the interaction between class I and TAP, the result lends credibility to any model in which the architecture of the F-pocket is central to understanding how the release of assembled class I molecules from the loading complex is achieved.

Concluding remarks

Defining the way in which newly synthesized MHC class I molecules interact with accessory molecules in the lumen of the ER is likely to provide the key to understanding how antigen presentation is regulated *in vivo*. To this end, several important questions should be addressed, such as how class I molecules are loaded with peptides *in vivo*, how this peptide-binding event brings about their release from the ER, and how incompletely assembled class I molecules are identified and disposed. In time, a full description of the intersecting pathways of antigen processing and class I assembly could provide a clear basis for therapeutic intervention in the context of cellular immune responses.

I am indebted to J. Torrno (University of Oxford) whose knowledge and insight added substance to a collection of vague ideas. I would also like to thank J. Lewis for his skills at the bench and for stimulating discussions, along with Y. Jones, P. Wood and J. Neefjes. T.J.E. is a Wellcome Trust Senior Fellow in Basic Biomedical Science.

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B-cell superantigens

Gregg J. Silverman

Superantigens (SAgs) are characterized by their ability to interact with T- and B-cell antigen (Ag) receptors outside of the classical Ag-binding groove. Here, Gregg Silverman discusses the molecular basis for the binding of B-cell SAgs, and how they may influence the acquisition of immunocompetence or contribute to the development of immune abnormalities associated with certain inflammatory and infectious diseases.

In recent years, several laboratories have described proteins that have unconventional immunoglobulin (Ig)-binding capacities that parallel the properties of known T-cell superantigens (SAgs). Among these proposed B-cell SAgs, the best characterized is the 42 kDa membrane protein of *Staphylococcus aureus* staphylococcal protein A (SpA) – which, by virtue of its Fab-binding activity, has become the prototype for a B-cell SAg. Other B-cell SAgs with similar binding characteristics include the gp120 envelope protein of certain isolates of human immunodeficiency virus 1 (HIV-1), and a human gut-associated sialoprotein termed protein Fv (pFv). In addition, Protein L from *Peptostreptococcus magnus* has the binding properties of a B-cell SAg but with a different type of Fab specificity[1].

The molecular basis for binding of a B-cell SAg

Minimal requirements for binding

In general, binding of a SAg is primarily dependent on the variable (V)-gene segment contribution of only one of the chains of the heterodimeric Ag receptor. In the best-characterized example, the Fab-binding site of SpA enables interactions with most Igs with heavy (H) chains from the human V_H3-gene family[2–7], but not Igs from other V_H families (reviewed in Ref. 8). This V_H-specific binding activity is distinct from the Fc-binding site of SpA (Refs 9, 10). Igs with Fab-mediated SpA-binding activity share conserved sequences in the framework 1 (FR1), FR3 and C-terminal complementarity determining region 2 (CDR2) that are diagnostic for V_H3 gene products[5,6], and competition studies indicate that these V_H3 Igs all interact

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The Protease Inhibitor, *N*-Acetyl-L-Leucyl-L-Leucyl-L-Norleucinal, Decreases the Pool of Major Histocompatibility Complex Class I–binding Peptides and Inhibits Peptide Trimming in the Endoplasmic Reticulum

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Summary

N-acetyl-L-leucyl-L-leucyl-L-norleucinal, (LLnL), which inhibits proteasomes in addition to other proteases, was found to prolong the association of major histocompatibility complex class I molecules with the transporters associated with antigen processing (TAP), and to slow their transport out of the endoplasmic reticulum (ER). LLnL induced a reversible accumulation of ubiquitinated proteins and changed the spectrum of peptides bound by class I molecules. These effects can probably be attributed to proteasome inhibition. Unexpectedly, in the TAP-deficient cell line .174, the rate of intracellular transport of human histocompatibility leukocyte antigen (HLA) A2 was also reduced by LLnL, and the generation of most HLA-A2–associated signal sequence peptides was inhibited. The inhibition of HLA-A2 transport in .174 cells was found to be less sensitive to LLnL than in wild-type cells, and a similar difference was found for a second protease inhibitor, benzyloxycarbonyl-L-leucyl-L-leucyl-L-phenylalanilal. These data suggest that under some conditions such inhibitors can block trimming of peptides by an ER peptidase in addition to inhibiting cytosolic peptide generation.

Newly synthesized MHC class I heavy chain β_2-microglobulin (β_2m)[1] dimers bind peptides in the endoplasmic reticulum (ER) before their transport to the cell surface. The vast majority of the associated peptides are derived from cytosolic proteins. These peptides are transported into the ER from the cytosol in an ATP-dependent fashion by the transporters associated with antigen processing (TAP; for a review see reference 1), which physically associate with peptide-free class I–β_2m dimers via the TAP.1 subunit (2–4). Peptide binding to the class I molecules triggers their release from TAP, allowing their transport to the cell surface. Certain MHC class I alleles also bind peptides derived from the signal sequences of a small number of secreted or type I transmembrane proteins (5–7). In these cases, peptide loading is usually, though not always (8), independent of TAP.

The predominant protease responsible for the generation of cytosolically derived, TAP-dependent, class I–associated peptides is thought to be the proteasome, a large (20S) multisubunit protease. The 20S proteasome can degrade proteins in vitro, but in vivo it predominantly exists as the nucleus of a larger (26S) ATP-dependent complex (9, 10). The 26S proteasome is responsible for the degradation of ubiquitinated proteins as well as at least one nonubiquitinated protein, ornithine decarboxylase (11, 12). Two subunits of the proteasome, LMP2 and LMP7, are encoded in the MHC (13). Mice with targeted disruption of either of these genes exhibit some deficiency in CTL development, and LMP7-deficient mice exhibit a reduction in expression of class I MHC molecules (14, 15). Additionally, treatment of antigen-loaded target cells with proteasome inhibitors prevents their recognition by class I–restricted CTL (16). It has also been argued that ubiquitination of cytosolic protein antigens is important for their recognition by CTL, because mutant cell lines temperature sensitive for a key step in ubiquitination exhibit reduced sensitivity to CTL (17), although this finding is not universally accepted (18).

One of the inhibitors used by Rock et al. (16) to implicate the proteasome in MHC class I peptide generation in living cells was the peptide aldehyde *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL), which has recently been

[1] *Abbreviations used in this paper:* β_2m, β_2-microglobulin; endo H, endoglycosidase H; ER, endoplasmic reticulum; IP-30, the γ interferon inductible protein; LLnL, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; RP, reverse phase; TAP, transporter associated with antigen processing; TBS, Tris-buffered saline; Z-LLF-CHO, benzyloxycarbonyl-L-leucyl-L-leucyl-L-phenylalanilal.

shown to bind to the active sites of the archebacterial proteasome (19). The mammalian proteasome has been shown to have at least five different proteolytic activities, and LLnL inhibits them to varying degrees (20, 21). In this study, we set out to examine the effects of proteasome inhibition on TAP–class I association and on the rate of egress of class I–peptide complexes from the ER. In addition to finding anticipated effects, we made the surprising observation that transport of HLA-A2 molecules in TAP-negative cells was slowed by LLnL, and by a second proteasome inhibitor, benzyloxycarbonyl-L-leucyl-L-leucyl-L-phenylalanilal (Z-LLF-CHO), and found that the profile of associated signal sequence peptides was also affected by LLnL. The implication of these findings for potential ER processing of class I–associated peptides is discussed.

Materials and Methods

Cell Lines. Transfectants of the HMY2.C1R cell line, C1R.A2, C1R.B7, and C1R.B27 and the TAP-negative mutant cell line .174 have been previously described (22, 23). All cell lines were maintained in IMDM (GIBCO BRL, Gaithersburg, MD) with 5% calf serum (Hyclone Laboratories Inc., Logan, UT) and gentamicin at 20 μg/ml.

Antibodies. The mAbs 4E (anti-HLA-B locus), BB7.2 (anti-HLA-A2), and 1G12 (antitransferrin receptor) were previously described (24–26). Affinity-purified anti-TAP.1 rabbit serum R.RING4C generated against a COOH-terminal peptide from TAP.1, (2), and the rat mAb 3B10.7 (anti–class I) were also previously described (27). Immunoblots were probed with a rabbit anti–ubiquitin serum generously provided by Dr. Arthur L. Haas (Medical College of Wisconsin, Milwaukee, WI).

Inhibitors. The protease inhibitor LLnL or Calpain Inhibitor 1 was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA) and prepared as a 25-mM (100×) stock solution in DMSO. The inhibitor Z-LL-F-CHO was also prepared in DMSO at 25 mM and was a kind gift from Dr. Marian Orlowski (Mount Sinai School of Medicine, New York, NY).

Metabolic Labeling. 8×10^6 cells were incubated in methionine-free medium containing 6% dialyzed FCS (Hyclone) with LLnL at 250 μM or the DMSO solvent as control, for 1 h at 37°C. The cells were pulse labeled with 0.5 mCi [^{35}S]methionine (ICN Biochemicals, Inc., Costa Mesa, CA) for 15 min in fresh methionine-free medium in the continued presence of LLnL or DMSO alone and chased with a 15-fold excess of unlabeled methionine at 37°C for the indicated times. Labeling was stopped by diluting the cells in cold PBS. For experiments involving reimmunoprecipitation, 2 mCi of [^{35}S]methionine was used.

Immunoprecipitations and Endoglycosidase H Treatment. Labeled cells were pelleted and lysed in 10 mM Tris, 150 mM NaCl (Tris-buffered saline [TBS]), pH 7.4, 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), or 1% digitonin (Wako Pure Chemical Industries, Ltd., Richmond, VA), containing 0.5 mM PMSF, 0.1 mM *N*-α-tosyl-L-lysyl-chloromethyl ketone (TLCK), and 5.0 mM iodoacetamide (IAA). Postnuclear supernatants were precleared for 1 h with normal rabbit serum and protein A–Sepharose and then incubated with 4E, BB7.2, or 1G12 and protein A–Sepharose for 1 h. Endoglycosidase H (endo H) digestions were performed as described previously (28). To detect TAP-associated class I molecules in R.RING4C immunoprecipitates from digitonin extracts, the protein A beads were heated at 100°C for 5 min in 2% SDS, 2 mM dithiothreitol in TBS, diluted 10-fold in 1% Triton X-100 in TBS with 10 mM IAA, and allowed to incubate at room temperature for 30 min. After cooling to 4°C, released class I heavy chains were precipitated as above with 3B10.7 and protein G–Sepharose.

Immunoblots. Blots were performed as described (29). Briefly, 10^6 cells were lysed in 100 μl 1% Triton X-100 in TBS as above. Postnuclear supernatants were diluted with reducing sample buffer, separated by a 5–20% gradient SDS-PAGE, and electroblotted onto an Immobilon membrane (Millipore Corp., Bedford, MA). The membrane was blocked for 1 h in PBS containing 0.05% Tween 20 and 5% dehydrated milk, rinsed in PBS, and incubated overnight at 4°C with the rabbit anti-ubiquitin serum diluted in PBS containing Tween 20 and dehydrated milk. Bands were visualized with horseradish peroxidase–conjugated secondary goat anti–rabbit IgG antibody and epichemiluminescence (ECL) substrate (Amersham Corp., Arlington Heights, IL).

Analysis of ^{3}H-labeled Class I–associated Peptides. Cells ($6–8 \times 10^8$) in log phase growth were washed in PBS and incubated for 1 h at 37°C in Leu-free, Lys-free RPMI-1640 (GIBCO BRL) at 10^7 cells/ml, supplemented with 3% dialyzed FCS and 10 mM Hepes (GIBCO BRL) with or without 25 mM LLnL, added as a 100× stock. Control experiments contained equal concentrations of DMSO (0.1% vol/vol). 1 mCi each of L-[3,4,5-^{3}H]leucine and L-[4,5-^{3}H]lysine (Amersham Corp.) was added to both cell solutions and incubated for 5.5 h at 37°C. Cells were washed with PBS and class I–associated peptides were isolated as previously described (6). Briefly, pellets were lysed at 10^7 cells/ml in 2% polyoxyethylene lauryl ether (Sigma Chemical Co.) in 10 mM Tris, 50 mM NaCl, pH 7.4, with PMSF, TLCK, and IAA. The postnuclear supernatants were cleared by centrifugation for 1 h at 100,000 *g* and applied to affinity columns. Affinity columns were packed with Biogel A15m beads (Bio-Rad Laboratories, Hercules, CA) coupled to the mAbs 4E or MA2.1. Bound class I molecules were eluted and denatured by adding 10% acetic acid, and low molecular weight species were separated from class I heavy chain and β_2m by filtering through a Centricon 10 (Amicon, Beverly, MA). Filtrates were resolved on a reversed-phase (RP) column (μBondapak C18) using a HPLC system (Waters Chromatography Division, Milford, MA). Gradients were generated using an increasing concentration of acetonitrile in 0.1% hydrochloric acid. Flow was 0.5 ml/min and 1.0-ml fractions were collected.

Quantitation of Gel Bands. The ratio of endo H–resistant to endo H–sensitive forms of class I was determined by exposing the dried SDS-PAGE gel to a low intensity phosphorus screen and scanning by a GS-250 Molecular Imager (Bio-Rad Laboratories). The bands were quantitated using the program Molecular Analyst Version 2.0.1 (Bio-Rad Laboratories) run on a Macintosh 8100/80 (Apple, Inc., Cupertino, CA).

Peptide Synthesis and Retention Times. Peptides used to determine the retention times of HLA-A2 signal sequences were synthesized and purified by the Keck Foundation Biotechnology Resource Laboratory (Yale University). Retention times were determined by loading and eluting 10 μg of each peptide individually using the column and gradient conditions described above.

Results

LLnL Causes a Reversible Accumulation of Ubiquitinated Proteins. Ubiquitin-dependent proteolysis is believed to be the major nonlysosomal proteolytic pathway (30, 31). Ubiq-

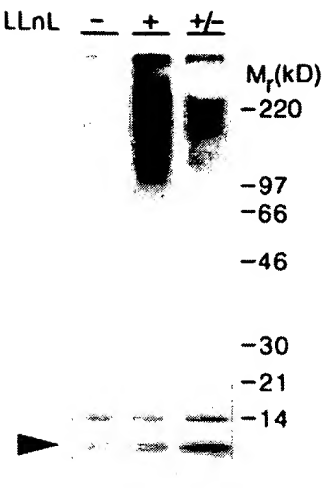

Figure 1. LLnL causes a reversible accumulation of ubiquitinated proteins. C1R.B27 cells were: incubated for 1 h in DMSO alone, washed, and incubated for another hour with DMSO (lane −); incubated with 250 μM LLnL, washed, and incubated again with LLnL (lane +); or incubated with LLnL, washed, and then incubated in DMSO only (lane +/−). Cell lysates were separated by a 5–20% SDS-PAGE gradient gel and blotted for ubiquitin using ECL. (*Arrowhead*) Position of monomeric ubiquitin.

uitinated proteins are targeted for degradation by the 26S protease complex, resulting in free ubiquitin and peptide fragments (11). To confirm that inhibition of the core 20S proteasome of this complex by LLnL disrupts the degradation of ubiquitinated proteins, cell lysates of LLnL-treated cells were subjected to SDS-PAGE, electrophoretically transferred to an Immobilon membrane, and probed with an anti-ubiquitin serum (Fig. 1). In control cells, the major bands were found at ~8 and 14 kD, most likely representing ubiquitin and di-ubiquitin, respectively. However, when cells were treated with LLnL, the majority of the anti-ubiquitin–reactive species migrated in the high molecular weight region, between 97 and 300 kD (Fig. 1, *center* lane). The species between 97 and 300 kD presumably represent a mixture of ubiquitinated proteins normally degraded by the 26S protease complex (32). The accumulation of ubiquitinated proteins was shown to be reversible. Cells washed free of LLnL and then incubated at 37°C for an additional hour (Fig. 1, *right* lane) exhibited a decrease in high molecular weight bands. Enhancement of the low molecular weight bands represents a large pool of newly freed ubiquitin and di-ubiquitin induced by LLnL. These results are consistent with the suggestion that a major target of LLnL is the proteasome.

LLnL Treatment Slows the Egress of MHC Class I Complexes from the ER. Inhibition of peptide generation by the proteasome would be expected to reduce MHC class I peptide loading, and consequently, delay class I transport. To examine this, C1R.A2, C1R.B7, and C1R.B27 cells were incubated in the presence or absence of 250 μM LLnL for 1 h at 37°C. In the continued presence or absence of inhibitor, the cells were pulse labeled with [^{35}S]methionine, chased for various times, and extracted in detergent. Class I molecules were immunoprecipitated with the conformation-specific antibodies BB7.2 (anti-HLA-A2) or 4E (anti-HLA-B). After treatment with or without endo H the immunoprecipitates were subjected to SDS-PAGE and the ratio of the endo H–resistant to endo H–sensitive class I molecules was quantitated (Fig. 2, *A–C*). Fig. 2 *A* shows that almost all of the HLA-A2 molecules had become resistant to endo H by 120 min in control cells. However, in the presence of LLnL, the rate of acquisition of endo H resistance was reduced. The amount of precipitable HLA-A2 was also decreased in cells treated with LLnL, as seen by the decreased band intensity. Both of these findings are consistent with a reduction in class I–associated peptides. HLA-A2 cannot leave the ER until peptide has bound, and the amount of stable, properly assembled class I molecules is decreased. Similar results were found with HLA-B7 and HLA-B27 (Fig. 2, *B* and *C*).

As a specificity control, a pulse–chase analysis of a non-peptide-dependent molecule, the transferrin receptor, was performed (Fig. 2 *D*). The transferrin receptor is a dimer of a 90-kD protein with three N-linked glycans. To accurately determine the kinetics of receptor egress from the ER, bands corresponding to the mobility of glycosylated and nonglycosylated transferrin receptor (filled and unfilled arrowhead, respectively; Fig. 2) were quantitated at each time point. No difference was seen in the rate of transport of the transferrin receptor with or without LLnL. Similar results were found for transferrin receptor transport with the cell line .174 and other C1R transfectants (data not shown).

LLnL Treatment Enhances MHC Class I Association with TAP.1. Peptide binding is believed to trigger the release of MHC class I molecules from TAP (2, 3). To determine if the inhibition of peptide generation by LLnL would prolong the association of class I HLA molecules with TAP proteins, pulsed and chased cells were solubilized in digitonin and the extracts were immunoprecipitated with an anti-TAP.1 antibody. Associated class I molecules were removed by SDS treatment, reprecipitated with an anti–class I heavy chain antibody (3B10.7), and separated by SDS-PAGE. The amount of class I associated with TAP.1 was both enhanced and prolonged in LLnL-treated cells (Fig. 3, *A–C*). This is most clearly seen at 240 min of chase, where almost undetectable levels of MHC class I molecules were TAP associated in control cells whereas clear bands were present in the LLnL-treated cells.

LLnL Affects Peptide Association with MHC Class I Molecules. To ascertain if peptide association with class I molecules was affected by treatment with LLnL, cells were metabolically labeled and the isolated peptides separated by RP-HPLC. Fig. 4, *A* and *B* depict peptides eluted from HLA-A2. The amount of stable HLA-A2 molecules recovered from cells treated with LLnL was decreased to less than half the amount recovered from control cells. As a result, the total yield of isolated peptides was reduced. The reduction in recovery was not a consequence of a reduction of overall labeling efficiency. LLnL had no effect on labeling efficiency under the conditions used. The population of peptides bound by HLA-A2 was also qualitatively different as evidenced by the general suppression of peaks with the exception of a single peak in fraction numbers 70-74 (Fig. 4 *B*). The most dramatic effect on bound peptides was seen with HLA-B7 (Fig. 4, *C* and *D*). In this example, equal numbers of cells were treated, and again, less than half the amount of class I molecules was recovered. Here the profiles were normalized to account for the difference in the amounts of recovered heavy chain and showed a striking

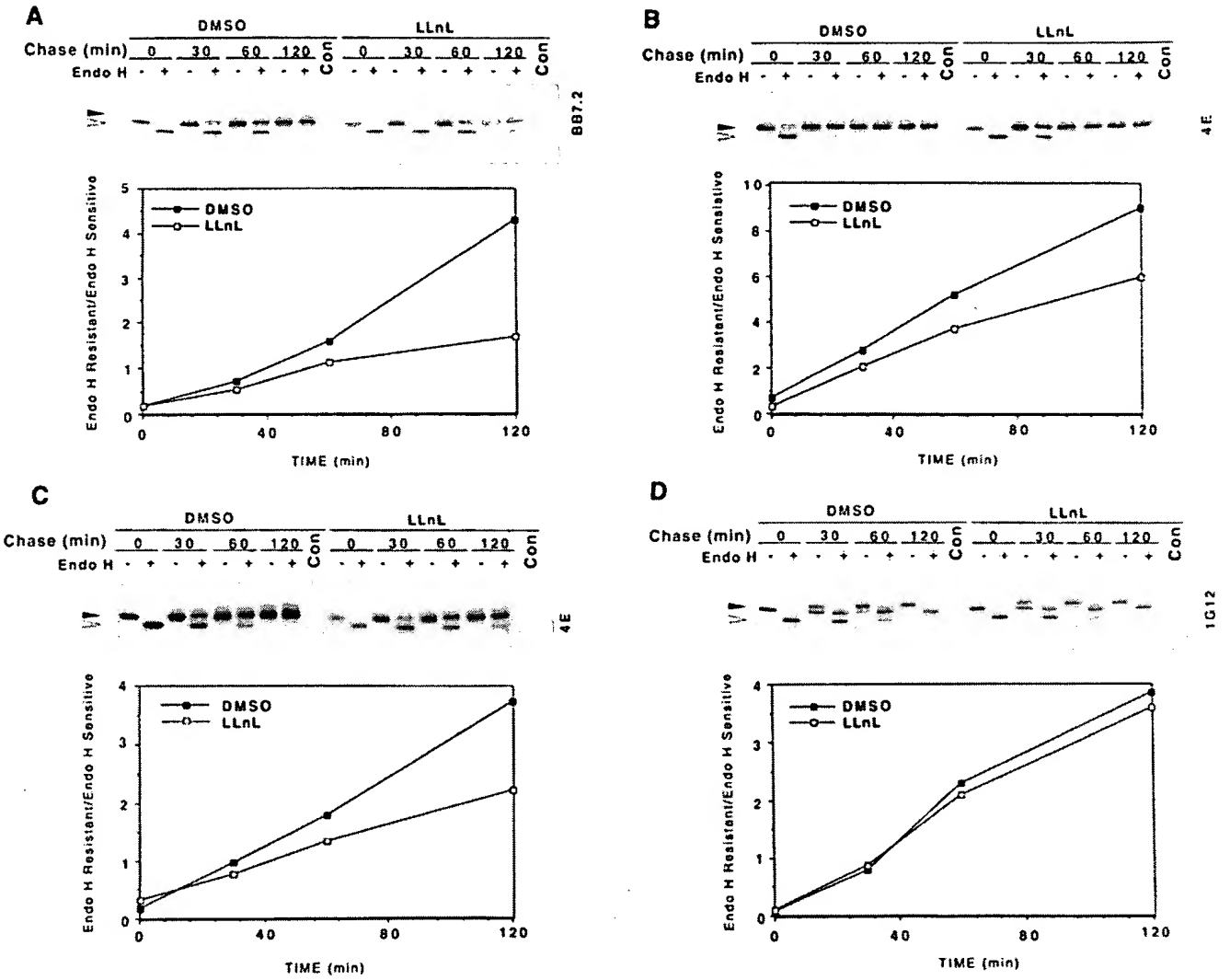

Figure 2. LLnL slows the transport of MHC class I molecules but not a peptide-independent molecule, the transferrin receptor. Cells were preincubated for 1 h with 250 μM LLnL or solvent alone at 37°C, pulsed for 15 min with 0.5 mCi of [^{35}S]methionine, and chased for 2 h. Immunoprecipitates were treated with endo H and were separated on a 10.5% SDS-PAGE gel. The ratio of endo H–resistant bands to endo H–sensitive bands are shown in the respective graphs. Cells and precipitating antibodies are as follows: (*A*) C1R.A2, BB7.2; (*B*) C1R.B7, 4E; (*C*) C1R.B27, 4E; and (*D*) C1R.B7, 1G12 (control, antitransferrin receptor). Controls (lanes *Con*) used isotype-matched antibodies. (*Filled arrowheads*) Endo H–resistant bands; (*unfilled arrowheads*) endo H–sensitive bands.

enhancement of peaks in fractions 100 and 125 in the LLnL-treated population (note the change in the y-axis). A similar but less dramatic effect was seen for HLA-B27–bound peptides, also normalized for the amount of recovered heavy chain (Fig. 4, *E* and *F*). For HLA-B27, as in the previous examples, peaks at 75, 90, and 110 were enhanced. The augmentation of a single peak in the presence of LLnL was also demonstrated with HLA-A3 (data not shown). The peptides that are enhanced by LLnL may represent peptides translated at this length in the cytoplasm requiring no proteolytic cleavage, they may be peptides whose generation is unaffected by the inhibitory action of LLnL, or they may be signal sequence peptides generated in the ER whose generation is also unaffected by LLnL. Both HLA-A2 and HLA-B7 have been shown to bind signal sequence–derived peptides (5–7). To determine the origin of these peptides, it would be necessary to sequence them. Unfortunately, the time during which cells can be treated with LLnL is limited to 10 h, after which they begin to die (data not shown). This would make it difficult to accumulate sufficient quantities of the peptides for sequencing.

LLnL Inhibits Peptide Generation in the ER. The prolonged TAP association and slower transport of MHC class I molecules in LLnL-treated cells was assumed to result from a reduction in the supply of cytosolically generated peptides. HLA-A2 in .174 and T2 cells binds signal sequence peptides, and its transport should therefore not be affected by LLnL. Unexpectedly, however, the rate of egress of HLA-A2 from the ER in .174 was found to be greatly diminished in the presence of the inhibitor (Fig. 5 *A*). To determine if the inhibi-

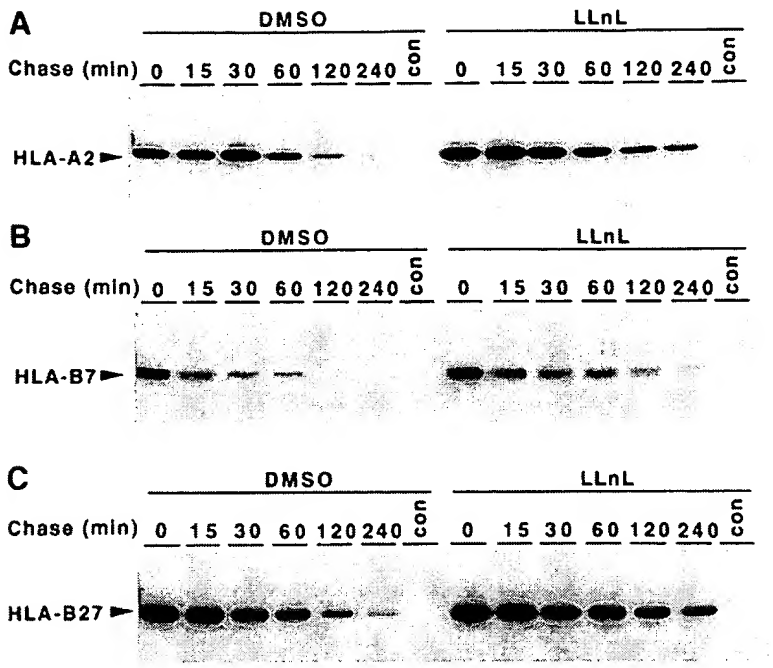

Figure 3. LLnL enhances and extends the association of MHC class I molecules with TAP 1. Cells were preincubated for 1 h in 250 μM LLnL or solvent alone at 37°C, pulsed for 15 min with 2.0 mCi of [^{35}S]methionine, chased for 4 h and extracted in 1% digitonin. TAP molecules were immunoprecipitated using purified anti-TAP. 1 rabbit antibodies. Associated class I heavy chains were released by SDS denaturation and reprecipitated using the mAb 3B10.7 (see Materials and Methods). Cells were as follows: (*A*) C1R.A2; (*B*) C1R.B7; and (*C*) C1R.B27.

tion of signal sequence peptide generation was reducing HLA-A2 assembly and transport, HPLC profiles of peptides bound by HLA-A2 in .174, with and without LLnL treatment, were generated (Fig. 5 *B*). The major doublet peak 3 and the minor peak 1 were decreased in the presence of LLnL, whereas peaks 2 and 4 were unaffected. These peaks, in the cell line T2 (a fusion product of .174 and similarly deficient in TAP expression), were previously found

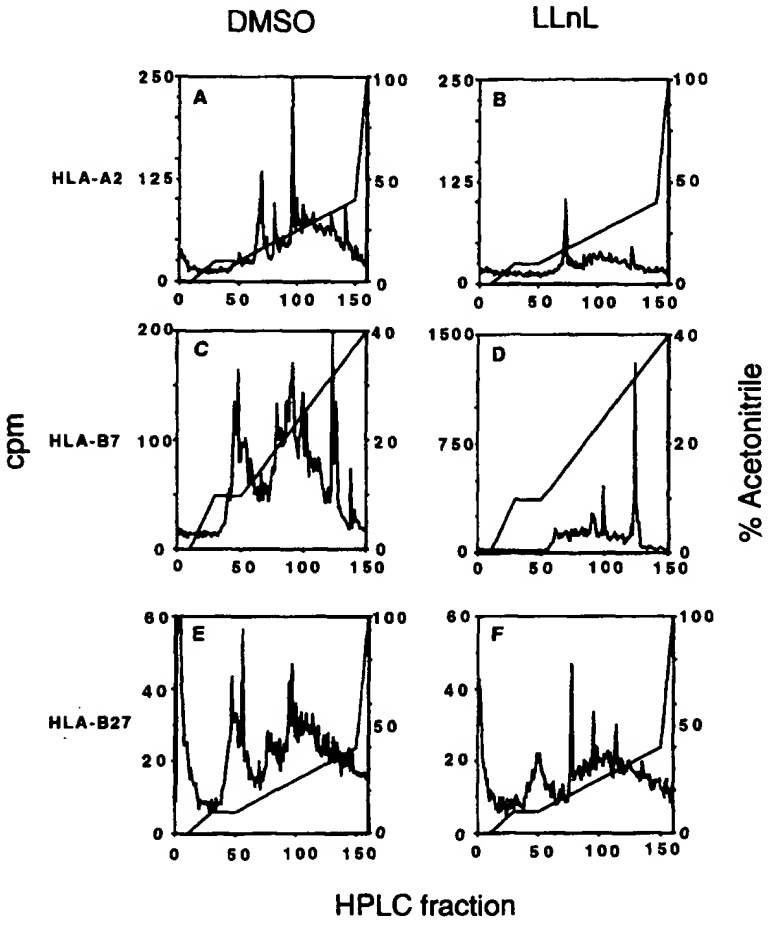

Figure 4. LLnL reduces the amount of assembled MHC class I molecules and changes the profile of peptides bound. Cells were preincubated with 250 μM LLnL or solvent alone for 1 h at 37°C, then labeled with 1 mCi each of L-[3,4,5-^{3}H]leucine and L-[4,5-^{3}H]lysine for 5.5 h. MHC class I molecules were isolated by affinity purification and the bound peptides were separated by HPLC (see Materials and Methods). Cells and affinity columns were as follows: (*A–B*) C1R.A2, MA2.1 (peptides loaded on HPLC were not normalized for class I recovery); (*C–D*) C1R.B7, 4E (peptides loaded were normalized for class I recovery); and (*E–F*) C1R.B27, 4E (peptides loaded were normalized for class I recovery).

to be peptides derived from signal sequence peptides (5, 6). The six signal sequence peptides isolated from HLA-A2 in T2 were synthesized, and their retention times in RP-HPLC were determined (Table 1). These peptides included fragments of the signal sequences of calreticulin, the γ-interferon inducible protein (IP-30), and the signal sequence receptor α subunit. The synthetic peptides coeluted with the peptides isolated from .174, although the requirement for collecting fractions to detect the ^{3}H-labeled peaks made it impossible to resolve three of the peptides, which are grouped as peak 3 in Fig. 5 and Table 1. However, only peaks 2 and 4 were unaffected by LLnL. Peak 2 corresponds to the longest peptide (12 residues) derived from IP-30 and terminates in a COOH-terminal glutamine residue. Peak 4 corresponds to a part of the calreticulin signal sequence and ends in glycine. Peak 1 and all the potential components of peak 3 terminate in valine or alanine. Thus LLnL in this case may be inhibiting a peptidase with specificity for an aliphatic residue. Because signal sequences are cleaved in the ER, and the TAP deficiency of .174 precludes the reentry of peptides into the ER after cytosolic trimming, it seems most likely that the affected enzyme is an ER peptidase.

Cytosolic and ER Proteolysis Have Distinct Sensitivities to LLnL and Z-LLF-CHO. To determine the relative sensitivities of the proteases affecting class I assembly in C1R.A2 and .174 to LLnL and a more potent proteasome inhibitor (Z-LLF-CHO; 20), we titrated their effects on the intracellular transport of HLA-A2 by pulse–chase analysis (Fig. 6). Inhibition of HLA-A2 transport by LLnL and Z-LLF-CHO in C1R.A2 was detectable at 2.0 and 0.08 μM, respectively (Fig. 6, *A* and *C*). However, in .174, 50.0 μM LLnL and 2.0 μM Z-LLF-CHO were required for an observable effect (Fig. 6, *B* and *D*). These findings are consistent with the idea that the responsible protease in C1R and the responsible protease inhibited in .174 are different, and that the protease in C1R, presumably the proteasome, is significantly more sensitive. Z-LLF-CHO was also found to affect the peptide profile of CIR.B7 in a similar fashion to LLnL (data not shown).

Discussion

Many studies have focused on the role of the 26S protease complex (and its core subunit, the 20S proteasome) in

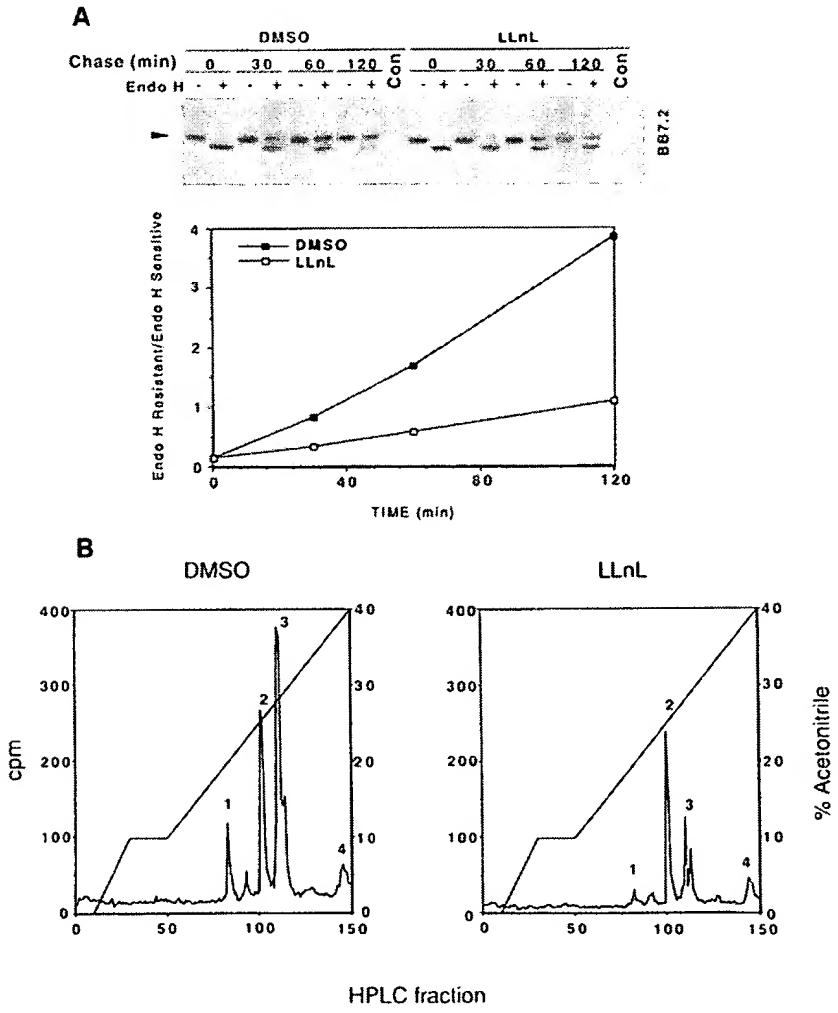

Figure 5. LLnL slows HLA-A2 transport through the Golgi in the TAP-negative cell line .174 and inhibits the generation of signal sequence peptides. 721.174 cells were preincubated in 250 μM LLnL or solvent alone, metabolically labeled for 15 min, and chased in the continual presence of inhibitor. (*A*) HLA-A2 was precipitated from detergent extracts of the cells harvested at the indicated times using the mAb BB7.2 and the ratios of endo H–resistant to endo H–sensitive class I determined; (*B*) .174 cells were treated with either 250 mM LLnL or solvent alone and labeled with 1 mCi each of L-[3,4,5-^{3}H]leucine and L-[4,5-^{3}H]lysine for 5.5 h. HLA-A2 was isolated using an MA2.1 affinity column and associated peptides were separated by RP-HPLC.

Table 1. *HLA-A2-associated Signal Sequence-derived Peptides*

Peak	Synthetic peptide sequence	Peptide source and reference	Retention time
			min
1	LLDVPTAAV	IP-30; Wei and Henderson	93.74
2	LLLDVPTAAVQ	IP-30; Wei	102.76
3	LLLDVPTAAVQA	IP-30; Henderson	105.43
	LLLDVPTAAV	IP-30; Henderson	107.51
	VLFRGGPRGLLAV	SSRα; Wei	107.82
4	MLLSVPLLLG	Calreticulin; Henderson	134.89

Retention times and source of HLA-A2–associated peptides in .174 (see Fig. 5). Indicated references are Wei and Cresswell (6) and Henderson et al. (5).

the generation of class I bound peptides. These studies have used LMP7 and LMP2 knockout mice (14, 15), cells expressing a temperature-sensitive ubiquitination phenotype (17, 18), and inhibitors of the proteasome (16). Proteasome inhibitors, many being peptide aldehydes, have been found to inhibit the proteolytic activity of the 20S proteasome in vitro (20, 21), to bind to the active site in the crystal structure of the 20S proteasome (19), and to block the generation of peptides from cytoplasmic proteins and prevent the subsequent expression of peptides on the cell surface in conjunction with MHC class I (16). To further characterize the effect of proteasome inhibitors on the processing and loading of MHC class I molecules, we investigated one of the most widely used cell-permeable inhibitors, LLnL.

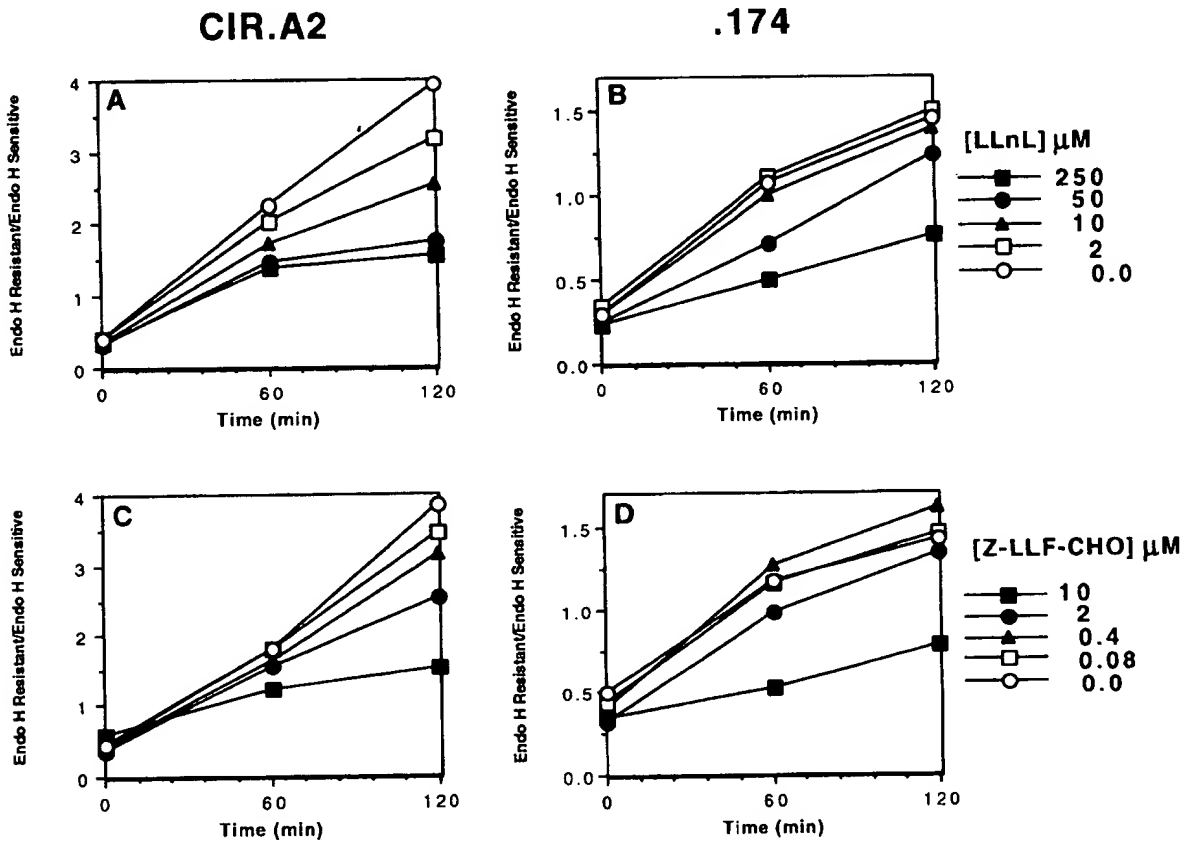

Figure 6. HLA-A2 transport in .174 is less sensitive to both LLnL and Z-LLF-CHO than in the wild-type cell C1R.A2. CIR.A2 (*A* and *C*) and .174 (*B* and *D*) cells were preincubated in the indicated concentration of LLnL (*A* and *B*) or Z-LLF-CHO (*C* and *D*), metabolically labeled for 15 min, and chased for 2 h in the continued presence of the inhibitor. HLA-A2 was immunoprecipitated with BB7.2 at the indicated times and the ratios of endo H–resistant to endo H–sensitive class I determined (see Materials and Methods).

LLnL treatment produced all of the predicted effects on class I processing that would result from a peptide-deficient state in the cell. First, the rate at which class I molecules were transported from the ER was slowed in the presence of LLnL. Second, the association of class I and TAP molecules in the ER was enhanced and extended by treatment with LLnL. Third, the amount of peptides bound by class I molecules was decreased. LLnL caused a reversible accumulation of ubiquitinated proteins, normally degraded by the 26S protease complex. Rock et al. (16) showed that the ability of a range of peptide aldehyde inhibitors, including LLnL, to inhibit proteasome function, correlated with their ability to block MHC class I–restricted antigen processing. Thus, although the precise role of ubiquitination in antigen processing remains in question, the combination of evidence strongly argues that the proteasome is the major protease involved.

In addition to reducing the overall yield of MHC class I molecules and associated peptides (Fig. 4, *A* and *B*), LLnL also induced changes in the profile of peptides bound. This is particularly evident in Fig. 4, *C* and *D*, where two HLA-B7–associated peaks are dramatically increased. These and similar peaks must correspond to peptides either unaffected by LLnL and better represented because of an overall reduction in the available competing pool of peptides, or to peptides actively enhanced by LLnL treatment. For example, polypeptides might normally be cleaved within the peptide sequences enhanced in LLnL-treated cells. This could occur either in the cytosol, perhaps mediated by the chymotryptic-like activity most strongly inhibited by LLnL (21), or even in the ER after TAP-mediated translocation.

That peptide cleavage can occur in the ER is clearly shown by the effects of LLnL on the signal sequence–derived peptides associated with HLA-A2 in the TAP-negative .174 cell line (Fig. 5). LLnL treatment unexpectedly slowed the egress of HLA-A2 from the ER in .174, as did a second inhibitor, Z-LLF-CHO (Fig. 6). The inhibitors had no effect on the transport rate of transferrin receptors in .174 cells, arguing for an effect specific to class I molecules (data not shown). HLA-A2 escapes the ER in TAP-negative cell lines because it binds peptides generated from a number of hydrophobic signal sequences. We found that LLnL inhibited the generation of the majority of signal sequence–derived peptides that bind to the HLA-A2 allele and propose that the decrease in HLA-A2–specific peptides reduces the number of properly assembled, transport-competent HLA-A2 molecules. Effects on signal sequence degradation are unlikely to result from proteasome inhibition because the proteasome is confined to the cytosol and nucleus (33). Although evidence exists for an ATP-dependent mechanism for peptide translocation from the ER to the cytosol (34), any peptides trimmed in the cytosol presumably would require TAP to reenter the ER. Thus, in .174, any peptidase involved in generating class I–associated peptides and affected by LLnL must reside in the ER. The data shown in Fig. 5 *B* and Table 1 suggest that the peptidase inhibited by LLnL may cleave COOH-terminal to aliphatic amino acids because the unaffected peptides (peak *2* and *4*, Fig. 5 *B*) terminate in a glutamate residue and glycine residue, respectively. However, with the limited number of peptides available for study it would be premature to make this a firm conclusion.

Transport of HLA-A2 molecules was found to be affected at lower concentrations of LLnL and Z-LLF-CHO in C1R cells than in .174 cells (Fig. 6). Nevertheless, it seems likely that some of the effects of LLnL on class I peptide loading in wild-type cells could result from inhibition of ER peptidases. When such inhibitors are used to investigate the origin of individual peptides that serve as T cell epitopes, as opposed to studies of the general process of MHC class I–restricted peptide generation, this possibility clearly must be borne in mind. Trimming of certain peptides in the ER, first suggested by Falk et al. (35) and for which reasonable evidence now exists (36, 37), may be inhibited by LLnL or other peptide aldehyde inhibitors that also inhibit proteasome-mediated degradation. Even more specific proteasome inhibitors, such as the recently described *Streptomyces*-derivative lactacystin (38), must be evaluated for possible effects on ER-mediated proteolysis before their use in antigen-processing studies can be properly evaluated. Clearly, inhibitors that specifically affect ER peptidases would be extremely useful in investigating the mechanisms involved in generating MHC class I–associated peptides. Effects on signal sequence degradation, measured using HLA-A2 in .174 cells to "trap"the degradation intermediates as shown in Fig. 5, might provide a useful assay for such inhibitors.

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Two Novel Routes of Transporter Associated with Antigen Processing (TAP)-independent Major Histocompatibility Complex Class I Antigen Processing

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Summary

Jaw1 is an endoplasmic reticulum (ER) resident protein representative of a class of proteins post translationally inserted into membranes via a type II membrane anchor (cytosolic NH_2 domain, lumenal COOH domain) in a translocon-independent manner. We found that Jaw1 can efficiently deliver a COOH-terminal antigenic peptide to class I molecules in transporter associated with antigen processing (TAP)-deficient cells or cells in which TAP is inactivated by the ICP47 protein. Peptide delivery mediated by Jaw1 to class I molecules was equal or better than that mediated by the adenovirus E3/19K glycoprotein signal sequence, and was sufficient to enable cytofluorographic detection of newly recruited thermostabile class I molecules at the surface of TAP-deficient cells. Deletion of the transmembrane region retargeted Jaw1 from the ER to the cytosol, and severely, although incompletely, abrogated its TAP-independent peptide carrier activity. Use of different protease inhibitors revealed the involvement of a nonproteasomal protease in the TAP-independent activity of cytosolic Jaw1. These findings demonstrate two novel TAP-independent routes of antigen processing; one based on highly efficient peptide liberation from the COOH terminus of membrane proteins in the ER, the other on delivery of a cytosolic protein to the ER by an unknown route.

MHC class I molecules bind peptides of 8–10 residues derived from intracellular proteolytic degradation and present them at the cell surface to $CD8^+$ T lymphocytes (T_{CD8+}) (1, 2). In the absence of high affinity peptide ligands, cell surface class I molecules are unstable at 37°C and rapidly denature (3). Such denaturation can often be detected by mAbs specific for the $\alpha1\alpha2$ domains: the binding of such mAbs to live cells provides a measure of the capacity of cells to produce class I molecules with stable peptide ligands.

The generation of the majority of class I–associated peptides involves cytosolic proteolysis. Little is known about how proteins are targeted in the cytosol for the production of class I–binding peptides. The nature of the proteases involved is only slightly better defined. The proteasome, an abundant, heterogeneous, macromolecular multicatalytic protease, has been implicated in the generation of a substantial portion of class I–binding peptides (4, 5). Other cytosolic proteases might also contribute to peptide generation, because proteasome inhibitors only partially block class I assembly and antigen presentation (6–9). Peptides of 8–16 or so residues produced by cytosolic proteolysis are transported into the endoplasmic reticulum (ER)[1] by the transporters associated with antigen processing (TAP), the MHC-encoded member of the ATP binding cassette transporter family of proteins (10–13). Longer peptides may also be transported, but at much reduced efficiency (14).

Functional TAP is required for the optimal assembly of class I molecules, as shown by the poor cell surface expression of class I molecules by TAP-deficient cells (15–18). This is due to absence of peptides in the ER, because delivery of peptides to the ER by appendage of a hydrophobic signal sequence can restore surface expression of class I molecules (19–22). Such peptides are thought to enter the ER by transiting the translocon, where signal peptidase lib-

[1] *Abbreviations used in this paper:* BFA, brefeldin A; ER, endoplasmic reticulum; FBS, fetal bovine serum; MOI, multiplicity of infection; NP, nucleoprotein; rVV, recombinant vaccinia virus; TAP, transporter associated with antigen processing; VV, vaccinia virus.

erates the class I–binding peptide from the hydrophobic signal sequence.

The ability of TAP to transport peptides longer than those usually recovered from class I molecules raises the possibility of peptide trimming in the ER, with peptide either free or bound to class I as originally proposed (23). Using TAP-deficient cells, it has been shown that class I–binding peptides can be liberated from longer precursors targeted to the ER via the translocon (24, 25). Peptide liberation occurs most readily from short precursors, but under some circumstances, class I–binding peptides can be derived from full-length proteins (26).

In the present study, we explore the capacity of ER-associated proteases to process antigenic peptides from the lumenal domain of Jaw1. Jaw1 is an ER resident protein whose known expression is limited to cells of hematopoietic origin (27). Jaw1 lacks a NH_2-terminal signal sequence, and is inserted into the membrane posttranslationally by a hydrophobic transmembrane region at residues 480–503 (28). Jaw1 consists of a large cytosolic domain of several coiled coils, the aforementioned transmembrane region, and a 35-residue lumenal tail (see Fig. 1). The membrane topology of Jaw1 and posttranslational insertion into the ER are representative of a number of integral membrane proteins (29). The membrane insertion of these proteins appears to occur independently of the translocon. In the course of investigating the antigen processing of a form of Jaw1 lacking the membrane anchor/insertion sequence, we unexpectedly encountered a novel route of delivery of antigenic peptides to class I molecules whose generation is dependent on a nonproteasomal activity.

Materials and Methods

Biochemical Procedures. T2 or L929 cells (2×10^6) were infected with recombinant vaccinia virus (rVV) for 3 h at 37°C, incubated for 30 min in 5 ml methionine-free DMEM (Biofluids, Rockville, MD) to deplete intracellular methionine pools. Infected cells were labeled with 50 μCi [^{35}S] methionine (Amersham, Arlington Heights, IL) in 200 μl methionine-free DMEM for 5 min. After washing in PBS containing 2 mg/ml free methionine (PBS/Met), cells were chased in IMDM for the indicated times. Cells were pelletted by centrifugation and suspended in lysis buffer (0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM Tris-HCl, pH 7.4, Complete™ protease inhibitor cocktail) (Boehringer Mannheim, Indianapolis, IN). Where indicated, cells were pretreated and proteasome inhibitors added at the concentrations described above. Lysates were collected with affinity-purified rabbit anti-Jaw polyclonal antibodies (28) conjugated to protein A–Sepharose (Pierce, Rockford, IL) for 2 h at 4°C with constant rotation. Beads were washed extensively and boiled in sample buffer containing 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol. Samples were analyzed by SDS-PAGE according to Laemmli (30). Gels were dried by vacuum and exposed to phosphorescent screens overnight and for 1 wk. Screens were imaged using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Images were prepared using Adobe Photoshop and printed with a Fujix Pictrography digital printer (Fuji Medical Systems, Stamford, CT).

Cell Lines. L929 cells (American Type Culture Collection, Rockville, MD) and L929 transfected with genes coding for the MHC molecules K^b (L-K^b) or D^b (L-D^b cells) were maintained in DMEM supplemented with 7.5% FBS. T2 cells (16) and their class I transfectants T2-K^k, T2-D^b were maintained in IMDM supplemented with 7.5% fetal bovine serum (FBS) (vol/vol). T2-K^d cells were maintained in RPMI-1640 supplemented with 7.5% FBS. All cell lines were incubated at 37°C, 91% Air, 9% CO_2.

Cytofluorography. T2-K^d cells were infected with rVV at 10 PFU/cell for 16 h at 37°C with gentle rotation. Cells were washed in ice-cold PBS containing 1% rabbit serum, and incubated with SF1.1.1 mAb conjugated to FITC (PharMingen, San Diego, CA) at a 1:10 dilution for 30 min at 0°C, washed extensively in ice-cold PBS, and analyzed with a FACScan® (Becton Dickinson, San Jose, CA). Cells were suspended in ethidium homodimer (Molecular Probes, Eugene, OR) (10 μg/ml), and analysis was restricted to viable cells (nonfluorescent in FL3 photomultiplier tube).

Electron Microscopy. T2 cells were infected with rVVs for 6 h. Cells were washed twice in PBS and fixed in 0.5% glutaraldehyde for 15 min at room temperature. Cells were washed twice in PBS and fixed in a mixture of 3% paraformaldehyde and 0.5% glutaraldehyde in PBS for 15 min at room temperature. The cells were washed in PBS and further processed for cryomicrotomy and immunolabeling essentially according to methods described by Tokuyashu (31). Frozen-thawed sections were indirectly labeled with a 1:500 dilution of rabbit anti-Jaw antiserum followed by 10-nm colloidal gold coated with protein A (Aurion, Wageningen, Netherlands). The ultrathin sections were examined with a Philips EM400 electron microscope.

Protease Inhibitors. cbz-LL-CHO, cbz-LLL-CHO, and cbz-LLF-CHO were synthesized as described (32). Lactacystin was purchased from E.C. Corey (Harvard University, Cambridge, MA). N-Ac-LLnL and N-Ac-LLnM were purchased from Calbiochem Novabiochem (La Jolla, CA). Other inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO).

Mice. 6–8-wk-old BALB/cByJ, CBA/J, and C57BL/6J mice were purchased either from the Jackson Laboratories (Bar Harbor, ME) or Taconic Farms (Germantown, NY). Mice were primed intravenously with rVVs (5×10^6 PFU) or intraperitoneally with PR8 (200HAU) in BSS/BSA and spleens taken at least 3 wk after priming.

Microcytoxicity Assays. Target cells (2×10^6) were infected with rVV (2×10^7 PFU) for 1 h in balanced salt solution, 0.2% BSA (BSS/BSA), followed by an additional 3-h incubation in IMDM. Cells were labeled with 20 μl IMDM with 10 μCi $Na^{51}CrO_4$ (Amersham, Arlington Heights, IL) for 1 h at 37°C and washed in IMDM. Target cells were suspended in IMDM and incubated with splenic effector cells for 6 h at 37°C. Effector cells were generated from splenocytes primed with rVV expressing full-length protein antigen or minigenes and stimulated in vitro by PR8-infected autologous spleen cells. Where indicated, brefeldin A (BFA) (Sigma Chemical Co.) was added to cells at 5 μg/ml and maintained throughout the remaining incubations. In experiments with peptidyl aldehyde inhibitors, target cells were pretreated with either 25 μM inhibitor or as indicated in the Fig. 6 legend for 30 min at 37°C. Inhibitors were present during the 4-h infection.

Peptide Extraction. Peptides were extracted in trifluoroacetic acid as previously described (24) with minor modifications. T2 cells (10^9) were coinfected with rVV expressing K^d or EC15K^d and rVV-expressing Jaw1 constructs at multiplicity of infection (MOI) of 10 for 1 h at 37°C. Cells were incubated at 37°C for an

additional 16 h in 100 ml IMDM. Cell pellets were lysed in 10 ml 0.1% trifluoroacetic acid, Dounce homogenized, and sonicated. Lysates were passed through Macrosep filters (Filtron Technology Corp., Northborough, MA) to collect material <3,000 kD. Filtrates were vacuum concentrated and resuspended in 500 μl PBS. Serial twofold dilutions were incubated with ^{51}Cr-labeled P815 cells in 50 μl for 2 h at 26°C. To maximize binding of exogenous peptides, P815 cells were cultured overnight at 26°C with 5 μg/ml human β_2-microglobulin (Sigma). Nucleoprotein (NP)-specific effector cells were added at 10:1 effector to target ratio and incubated an additional 6 h. Percent-specific lysis was calculated as described above.

Viruses. The influenza virus A/PR8/(H1N1) (PR8) was grown in 10-d-old embryonated chicken eggs and used as infectious allantoic fluid. Recombinant VV were grown in thymidine kinase minus (TK$^-$) human 143B osteosarcoma cells. rVV expressing NP, SNP, cytosolic and ER-targeted peptides have been described (20, 33). VV-ICP47 (34) and VV-Kex2 (35) were provided by B. Rouse (University of Tennessee, Knoxville, TN) and D. Thomas (University of Oregon, Eugene, OR), respectively. Jaw1[$NP_{147-155}$] and Jaw1 (Lum$^-$)[$NP_{147-155}$] were engineered by PCR of the full-length Jaw1 mouse cDNA using the 5′ primer CTATTAGGTGACACTATAGAACAGACACCATGGCTCTCTGTGTAAAAGGT-CCC with unique 3′ primers GGGGTACCTCATCACACTAGTGCTCGTGTTCGCTGGTATGTAGCCTCCACGGCTGTCTG (Jaw1(Lum$^-$)[$NP_{147-155}$] and GGGTACCTCATCAC-CTAGTGCTCGTGTTCGCTGGTATGTTCGTTTCGTCGCACTGGCGGTGGTCCATC (Jaw1[$NP_{147-155}$]). The constructs contain a β-globin leader sequence upstream of the initiator ATG of Jaw1. Jaw1(Lum$^-$) [$NP_{147-155}$] has the $NP_{147-155}$ peptide TYQRTRALV fused in-frame after amino acid Ala509 and is followed by two stop codons. Jaw1[$NP_{147-155}$] has the Kex2/furin protease site RRKR and the peptide TYQRTRALV fused in-frame following the penultimate Val539 of the Jaw1 cDNA. Both PCR fragments were cloned blunt-ended into the SmaI site of pSP72 (Promega), digested with SalI and KpnI, and then subcloned into pSC11. Jaw1(TM$^-$)[$NP_{147-155}$] was engineered by first amplifying the COOH-terminal half of the Jaw1 cDNA using an internal primer 5′ GGGCTGGTGTCAGGCATG and a 3′ primer GGGGTACCTCATCACACTAGTGCTCGTGTTCGCTGGTATGTGACCCAGGAAGCCACTGA, which placed $NP_{147-155}$ in-frame with Jaw1 following amino acid Val467 (upstream of the transmembrane domain). This fragment was digested with NaeI and KpnI and then subcloned into the same sites of the Jaw1(Lum$^-$)[$NP_{147-155}$] plasmid, replacing the COOH-terminal coding region of that plasmid. The [$NP_{147-155}$]Jaw1 construct used the following primers in PCR of the Jaw1 cDNA: 5′-GATCGTCGACAAACAGACACCATGACATACCAGCGAACACGAGCACTAGTGCTCTGTGTAAAAGGTCCC and 3′-CATTGAGCTGCACGTCAGTCA. This construct fuses the immunogenic peptide in frame immediately following the initiator ATG of full-length Jaw1. The PCR fragment was digested with SalI and BamHI and cloned into pSP72 before shuttling into pSC11 and pSC65. All constructs were sequenced to ensure fidelity of PCR.

Results

Characterization of Jaw1-expressing rVVs. To study the ability of Jaw1 to deliver an antigenic peptide to the ER, we produced rVVs encoding the $NP_{147-155}$ peptide at the COOH terminus of either intact Jaw1 (Jaw[$NP_{147-155}$]), or truncated

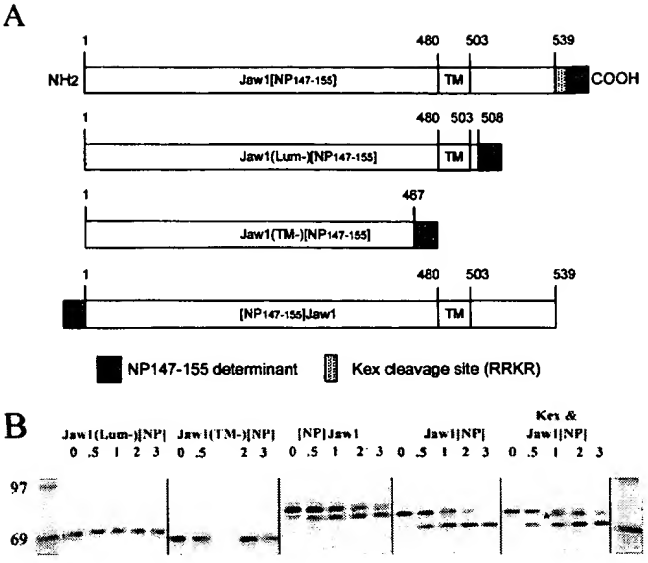

Figure 1. (*A*) Schematic representation of rVV expressed Jaw1 chimeric proteins. (*B*) Biochemical characterization of Jaw1 chimeric proteins. Species reactive with anti-Jaw1 antisera present in detergent extracts of rVV-infected cells pulse radiolabeled and chased for the indicated time in hours were analyzed by SDS-PAGE and visualized using a PhosphorImager. Only the region containing the antibody-reactive species is shown. The far left lane contains molecular weight markers indicated. The asterisk identifies a proteolytic fragment created by the action of Kex2 on Jaw1[$NP_{147-155}$].

versions lacking the lumenal domain (Jaw1(Lum$^-$)[$NP_{147-155}$]), or both the lumenal and transmembrane domains (Jaw1 (TM$^-$)[$NP_{147-155}$]). In designing Jaw1[$NP_{147-155}$] we inserted an additional tetrapeptide sequence at the peptide–Jaw1 interface that enables cleavage by the yeast Kex2 protease. We also produced a rVV-expressing Jaw1 with the $NP_{147-155}$ peptide appended to the NH_2 terminus ([$NP_{147-155}$] Jaw1) that serves as a topological control for the COOH-terminal $NP_{147-155}$-expressing chimeras. The various Jaw1 chimeric molecules are depicted schematically in Fig. 1 *A*.

Jaw1-containing gene products were initially characterized by SDS-PAGE of material collected with polyclonal rabbit anti-Jaw1 antibodies from [^{35}S]methionine-labeled detergent extracts prepared from rVV-infected cells (Fig. 1 *B*). Cells were radiolabeled for 5 min at 37°C and chased for up to 3 h at this temperature. In previous studies, we observed that Jaw1 migrates in SDS-PAGE with apparent mobility 10 kD greater than predicted (27). VV-encoded Jaw1 similarly migrates more slowly than predicted. This may be due to posttranslational modifications, but any such modifications would have to be rapid and complete because pulse-labeled material migrated as a single species. Therefore, it is more likely that the protein migrates aberrantly in SDS-PAGE. None of the Jaw1 chimeric proteins are N-glycosylated as determined by unaltered mobility after endoglycosidase H digestion (data not shown).

As anticipated, Jaw1 (TM$^-$)[$NP_{147-155}$] migrated most rapidly, followed by Jaw1(Lum$^-$)[$NP_{147-155}$]. Neither of these proteins was detectably modified over the 3-h chase

Figure 2. Immunogold localization of Jaw1 proteins. Jaw1 was located in rVV-infected T2 cells by cryoimmunogold labeling. (*A*) Cells expressing Jaw1 (Lum$^-$)[NP$_{147-155}$]. Gold particles specifically decorate the nuclear membrane and cytoplasmic membrane structures. (*B*) Cells expressing Jaw1 (TM$^-$)[NP$_{147-155}$]. Gold particles are located in the cytosol. In addition to the difference in patterns between *A* and *B*, the specificity of Jaw staining was shown by the absence of gold particles on sections prepared from cells infected with a control rVV.

period. Jaw1[NP$_{147-155}$] migrated with an apparent molecular mass of 4 kD greater than Jaw1(Lum$^-$)[NP$_{147-155}$], which is consistent with the addition of a 42-residue lumenal domain. [NP$_{147-155}$]Jaw1 migrated 3 kD more slowly than Jaw1(Lum$^-$)[NP$_{147-155}$]. Sequencing of the 5′ and 3′ ends of all of the genes revealed the expected sequences, so we again attribute the different mobilities to nonclassical SDS-PAGE behavior. During the chase period, [NP$_{147-155}$] Jaw1 and Jaw1[NP$_{147-155}$] were converted into more rapidly migrating forms. We previously showed this was likely due to proteolysis of the COOH terminus (28). By contrast, Jaw1(Lum$^-$)[NP$_{147-155}$] and Jaw1(TM$^-$)[NP$_{147-155}$] were not detectably cleaved over the 3-h chase period. This confirms the absence of lumenal domains in these proteins. The function of the Kex2 protease site was confirmed by coinfection of cells with VV-Jaw1[NP$_{147-155}$] and a rVV that directs the synthesis of Kex2, a secretory protease expressed at limiting levels by most cells (35). This resulted in the presence of a novel species migrating slightly faster than the pulse-labeled material (designated by an asterisk in Fig. 1 *B*), consistent with the removal of a small terminal peptide.

The intracellular targeting of two of the recombinant Jaw1 constructs was examined by cryoimmuno electron microscopy using colloidal gold-conjugated secondary antibodies to detect binding of anti-Jaw1 antibodies (Fig. 2). Almost all Jaw1(Lum$^-$)[NP$_{147-155}$] detected was associated with intracellular membranes, whereas Jaw1(TM$^-$)[NP$_{147-155}$] localized to the cytosol and was not specifically associated with membranes. Immunofluorescence of fixed and permeabilized cells with the anti-Jaw1 antiserum confirmed the expected location of each of the four Jaw1-chimeric proteins (data not shown).

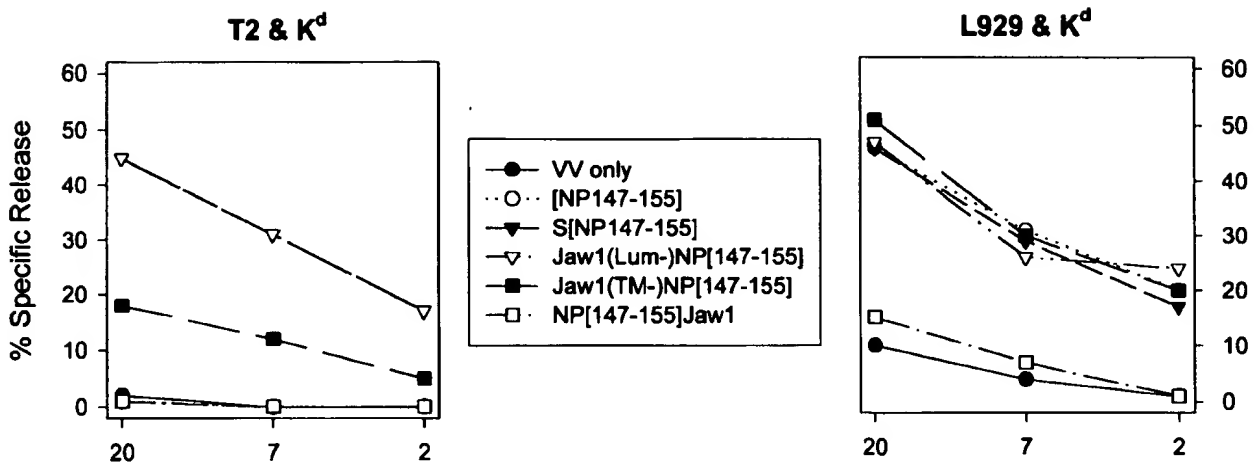

Figure 3. Presentation of $NP_{147-155}$ by rVV-infected cells. rVV-infected cells expressing the Jaw chimeric proteins were tested for lysis by NP-specific secondary T_{CD8+} at the indicated effector to target ratio. Cells were coinfected with a rVV expressing K^d to enable recognition.

Liberation of $NP_{147-155}$ from Jaw 1 Constructs: Antigen Presentation. The antigen processing of Jaw1 constructs was studied in T2 cells (a TAP-deficient human lymphoid cell line) or L929 cells (a TAP-expressing mouse cell line). To enable recognition by K^d-restricted, NP-specific T_{CD8+}, cells were coinfected with a rVV expressing K^d in addition to the Jaw1-expressing rVV. As seen in Fig. 3, L929 cells presented $NP_{147-155}$ from the COOH termini of both Jaw1(Lum$^-$)[$NP_{147-155}$] and Jaw1(TM$^-$)[$NP_{147-155}$]. Presentation from the NH_2 terminus of Jaw1 occurred at much lower levels. Because a Met-initiated cytosolic minigene product that is identical to the 10 NH_4-terminal residues of [$NP_{147-155}$]Jaw1 is effectively presented by L929 cells (designated in Fig. 3 and [$NP_{147-155}$]), this indicates that the Jaw1-derived COOH-terminal flanking sequences cannot be efficiently removed from $NP_{147-155}$ by cytosolic proteases. This compromises the use of this construct as a control in T2 cells, but only partially, because the activities of cytosolic proteases and secretory proteases are expected to be independent.

In T2 cells, both Jaw1(Lum$^-$)[$NP_{147-155}$] (Fig. 3) and Jaw1[$NP_{147-155}$] (data not shown) were efficiently presented after short VV infections (<4 h). This cannot be attributed to leakiness of the cells for cytosolic peptides, because under the same conditions, the cytosolic minigene, that is produced in enormous amounts relative to the amount of peptide liberated in the cytosol from proteins (36), was not presented above background values obtained with a control VV. [$NP_{147-155}$]Jaw1-infected cells were recognized by NP-specific T_{CD8+} at levels observed using cells infected with control rVVs. This is consistent with the predicted topology of Jaw1, but we cannot eliminate the possibility that the secretory pathway, like the cytosol, is incapable of liberating the $NP_{147-155}$ peptide from the NH_2 terminus of Jaw1. $NP_{147-155}$ was liberated from Jaw1(TM$^-$)[$NP_{147-155}$], but at far lower efficiency than from the transmembrane domain containing molecules (see Figs. 4 and 5).

Liberation of $NP_{147-155}$ from Jaw1-constructs: cytofluorography. We recently found that after infection of T2-K^d cells with rVVs encoding ER-targeted class I–binding peptides, enhanced K^d cell surface expression can be cytofluorographically detected after staining with K^d-specific mAbs (37). This provides a much more quantitative measure of the efficiency of antigen presentation than T_{CD8+}-mediated lysis. As previously observed, infection with a rVV expressing ER-targeted $NP_{147-155}$ resulted in a clear increase in the amount of mAb-reactive cell surface K^d on the surface of T2-K^d cells (Fig. 4). Remarkably, the greatest increase in

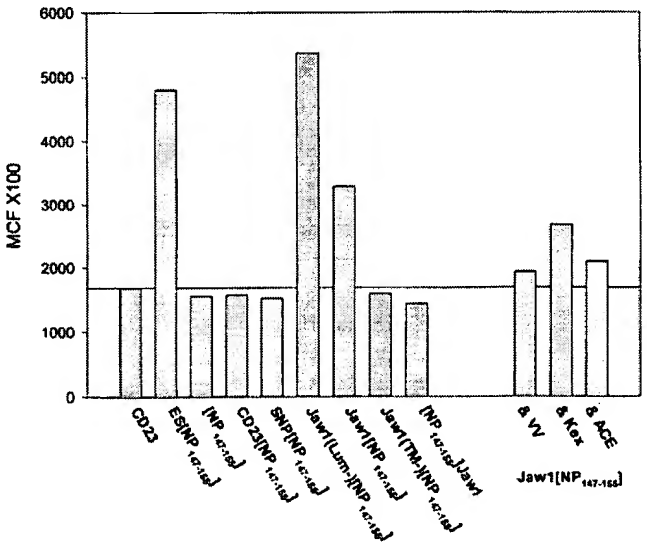

Figure 4. Rescue of K^d cell surface expression. T2-K^d cells were infected with the rVV indicated and the amount of cell surface K^d present on viable cells cytofluorographically determined using a directly conjugated K^d-specific mAb. Data are expressed as mean channel fluorescence × 100. The last three bars on the right represent cells coinfected with Jaw1[$NP_{147-155}$] and the rVV indicated (ACE, angiotensin-converting enzyme).

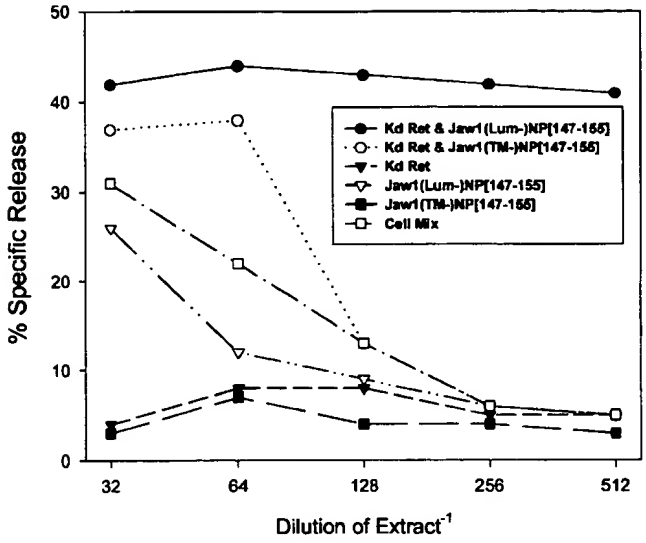

Figure 5. Quantitation of acid-soluble peptides in cell extracts. Extracts prepared from cells expressing ER retained K^d (*Kd Ret*) and the indicated Jaw1 chimeric protein were tested for their ability to sensitize P815 cells for lysis by NP-specific T_{CD8+}. Cell mix refers to a sample in which ER-retained K^d expressing cells were mixed with Jaw1(Lum$^-$)[$NP_{147-155}$]-expressing cells before lysis.

K^d expression was observed after infection with VV-Jaw1 (Lum$^-$)[$NP_{147-155}$]. K^d was rescued slightly less efficiently by expression of Jaw1[$NP_{147-155}$]. These effects can be attributed to the presence of the $NP_{147-155}$ peptide at the COOH terminus of Jaw1, because expression of [$NP_{147-155}$] Jaw1 had no effect on K^d expression. Thus, peptides generated from Jaw1 sequences do not themselves bind K^d and rescue expression. These findings demonstrate that Jaw1 efficiently delivers peptides to the class I processing pathway. In contrast with membrane-targeted Jaw1, expression of Jaw1(TM$^-$)[$NP_{147-155}$] had no effect on K^d expression. This demonstrates the importance of proper insertion of Jaw1 [$NP_{147-155}$] into the membrane to enable efficient peptide liberation in the secretory pathway.

In this experiment, we also examined whether liberation of $NP_{147-155}$ from Jaw1[$NP_{147-155}$] could be enhanced by coexpression of the yeast Kex2 protease encoded by a rVV. Because this entails coinfection with two rVVs, it was necessary to control for competition between rVVs for gene expression. Coinfection with VV reduced the Jaw1[$NP_{147-155}$]-mediated rescue of K^d expression. Above this baseline value, Kex2 coexpression clearly increased K^d rescue, whereas a rVV expressing the secretory carboxypeptidase angiotensin-converting enzyme had little effect on K^d expression, demonstrating the specificity of Kex2-mediated enhancement. These findings indicate that it is possible to modify the antigen processing of Jaw1 by coexpressing a protease.

$NP_{147-155}$ Liberated from Jaw1 Binds K^d in the ER of T2 Cells. To examine the site of liberation of $NP_{147-155}$ from Jaw1, we extracted peptides from cells coinfected with the Jaw1 expression rVVs and VV-EC15K^d. EC15K^d is a modified K^d molecule retained in the early secretory pathway by replacement of the normal cytosolic domain with that of E3/19K (38). The low relative molecular mass peptides present in trifluoroacetic acid homogenates prepared from infected cells were used to sensitize P815 cells for lysis by NP-specific T_{CD8+}. As seen in Fig. 5, antigenic peptides were recovered from cells expressing either Jaw1(Lum$^-$) [$NP_{147-155}$] or Jaw1(TM$^-$)[NP_{147}]. At least eightfold greater quantities of peptide were recovered from Jaw1(Lum$^-$) [$NP_{147-155}$] expressing cells than from Jaw1 (TM$^-$)[$NP_{147-155}$]-expressing cells. In an additional experiment in which we reached an endpoint with all of the lysates, we recovered ~40-fold more $NP_{147-155}$ activity from cells expressing Jaw1(Lum$^-$)[$NP_{147-155}$] than those expressing Jaw1(TM$^-$) [$NP_{147-155}$]. These findings are consistent with data in Fig. 4, in demonstrating that ER-targeted Jaw1 is a much more efficient vehicle for TAP-independent loading of K^d molecules with $NP_{147-155}$ than cytosolic Jaw1. Peptide recovery from Jaw1(TM$^-$)[NP_{147}]-expressing cells absolutely required coexpression of EC15K^d, whereas recovery from cells expressing Jaw1(Lum$^-$)[$NP_{147-155}$] was largely, but not entirely dependent on the EC15K^d expression (<5% of peptides were recovered in a K^d-independent manner). This small K^d-independent population of peptides may derive from endogenously expressed human class I molecules by T2 cells that weakly bind the peptides. Alternatively, the peptide (or a precursor that can be processed by cell surface of serum proteases in the course of sensitizing target cells), may be produced/stabilized in a class I–independent manner in the secretory pathway. To demonstrate that peptide association with K^d occurred before lysis of the cells, cells expressing VV-EC15K^d were mixed with VV-Jaw1(Lum$^-$) [$NP_{147-155}$]-infected cells and then lysed (Fig. 5, Cell Mix). This resulted in only a slight increase in the amount of peptide present in the extracts relative to VV-Jaw1(Lum$^-$) [$NP_{147-155}$]-infected cell lysates, demonstrating that the vast majority of $NP_{147-155}$ liberated from Jaw1(Lum$^-$)[$NP_{147-155}$] associates with K^d intracellularly. Based on these findings, we conclude that $NP_{147-155}$ is produced from both Jaw1(TM$^-$) [$NP_{147-155}$] and Jaw1 (Lum$^-$)[$NP_{147-155}$] in the early secretory pathway of T2 cells, probably the ER itself.

$NP_{147-155}$ Is Liberated from Jaw1(TM$^-$)[$NP_{147-155}$] by a Novel Protease Activity. The unexpected presentation of $NP_{147-155}$ from Jaw1(TM$^-$)[NP_{147}] in T2 cells hinted at an unusual mechanism of peptide entry into the class I presentation pathway. The cellular localization of Jaw1(TM$^-$)/ $NP_{147-155}$ suggested cytosolic proteases may participate in liberating NP peptide from Jaw1(TM$^-$). Peptide aldehyde inhibitors have been used to characterize protease activities that contribute to the production of class I–binding peptides. We initially studied the effect of the peptide aldehyde inhibitor cbz-LLL-CHO on the liberation of $NP_{147-155}$ from different precursors in T2 cells. cbz-LLL-CHO blocks all of the known activities of 20S proteasomes in vitro, and causes the accumulation of the ubiquitinated proteins in vivo (4, 32). T2 cells were treated with cbz-LLL-CHO before infection and throughout the 4-h incubation period before the ^{51}Cr release assay. At this time, cbz-LLL-CHO was replaced with BFA to prevent further

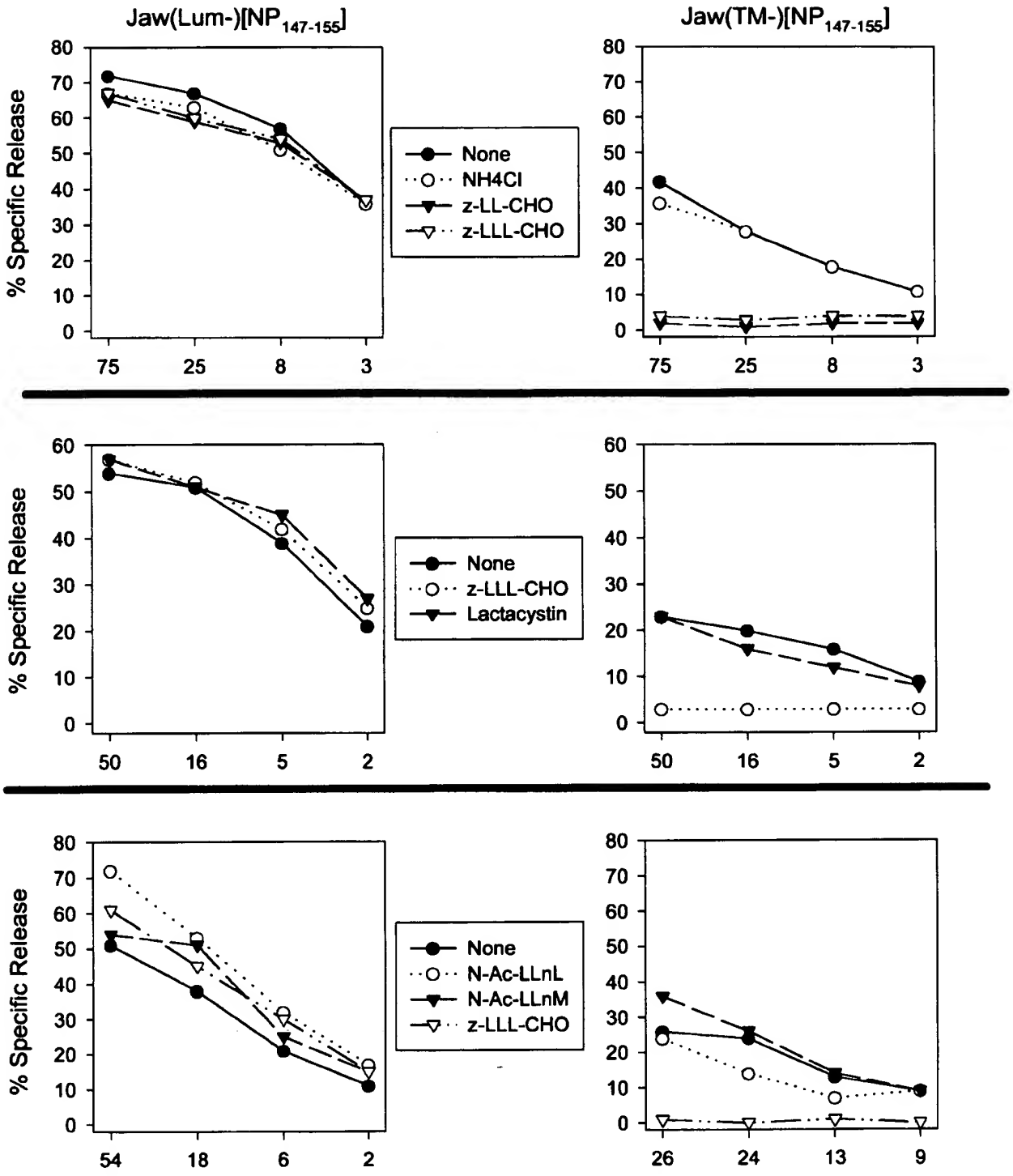

Figure 6. Effect of protease inhibitors on generation of the $NP_{147-155}$ peptide. rVV-infected T2-K^d cells expressing the indicated protein were incubated with the indicated protease inhibitor starting 30 min before infection and ending 4 h after infection. BFA was then added to cells to prevent additional antigen presentation and cells were incubated with $NP_{147-155}$-specific T_{CD8+} in a standard microcytotoxicity assay at the effector to target ratios indicated. Inhibitors were used at the following concentrations: NH_4Cl, 25 mM; cbz-LL-CH, 12.5 μM; cbz-LLL-CHO 12.5 μM (*A* and *B*), 25 μM (*C*); lactacystin, 10 μM; N-Ac-LLnL, 200 μM; N-Ac-LLnM, 200 μM. The three panels are derived from separate experiments.

delivery of class I–peptide complexes produced after the removal of cbz-LLL-CHO.

Using cbz-LLL-CHO at concentrations 12.5 or 25 μM did not detectably affect the liberation of $NP_{147-155}$ from Jaw1(Lum$^-$)[$NP_{147-155}$] (Fig. 6). By contrast, recognition of Jaw1(TM$^-$)[NP_{147}] expressing cells was dramatically reduced by cbz-LLL-CHO at either concentration. The effects of cbz-LLL-CHO cannot be attributed to blocking viral gene expression as demonstrated by cytofluorographic analysis of cells infected with a rVV-expressing mouse

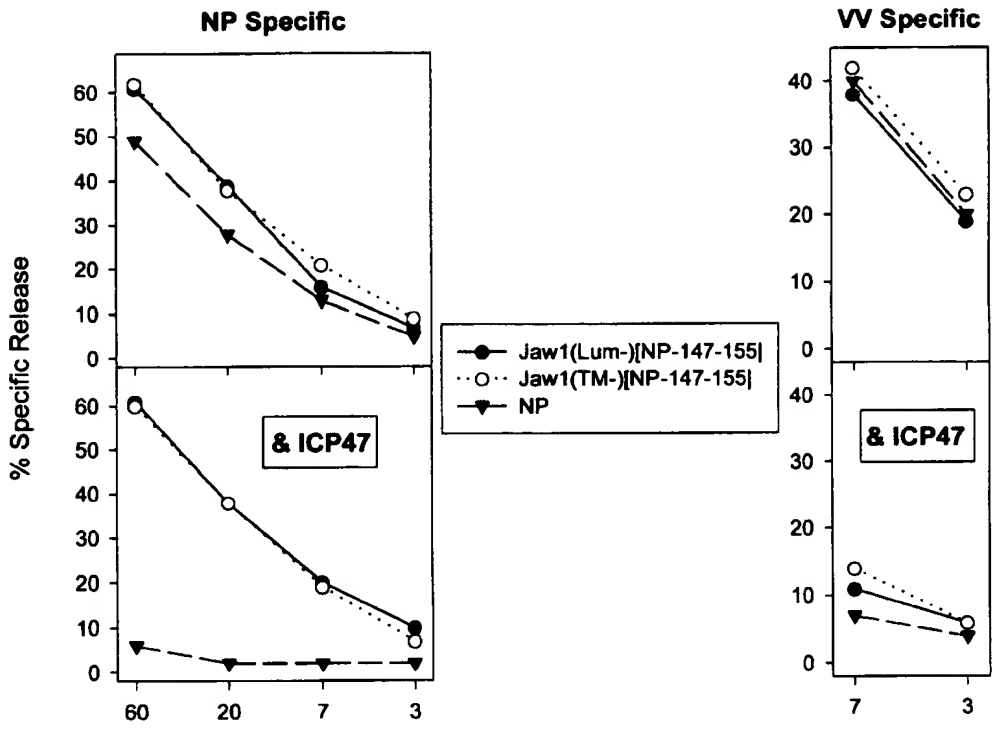

Figure 7. Tap-independent presentation in nonlymphoid cells. HeLa cells coinfected with rVVs expressing K^d, a Jaw1 chimerical protein, and either ICP47 or no foreign protein were tested for recognition by T_{CD8^+} populations specific for NP or VV at the indicated effector to target ratio.

ICAM 1. Cell surface levels of ICAM detected with saturating amounts of a directly conjugated mAb were not altered by cbz-LLL-CHO under the conditions used for studying antigen presentation (data not shown).

In addition to its effects on proteasomes, cbz-LLL-CHO inhibits other cytosolic and lysosomal proteases. To determine the nature of the target protease, we examined the effects of five additional protease inhibitors on the liberation of $NP_{147-155}$ in T2 cells (Fig. 6). None of the inhibitors blocked presentation of $NP_{147-155}$ by Jaw1(Lum$^-$)[$NP_{147-155}$]-expressing cells, indicating that global effects antigen presentation or viral gene expression are minimal and, therefore, that any effects on presentation of Jaw1(TM$^-$)[NP_{147}]-expressing cells are due to specific blockade of determinant liberation. Two of the additional inhibitors tested, cbz-LLF-CHO (data not shown) and cbz-LL-CHO blocked the liberation of $NP_{147-155}$ from Jaw1(TM$^-$)[NP_{147}]. cbz-LLF-CHO inhibits both proteasomes and other cellular proteases, whereas cbz-LL-CHO does not detectably affect proteasome activity at the concentration used (39), suggesting that the effects of peptide aldehyde inhibitors on presentation of Jaw1(TM$^-$)[NP_{147}] are due to blockade of nonproteasomal proteases. This was confirmed by the failure of lactacystin to affect presentation of Jaw1(TM$^-$)[NP_{147}]. Lactacystin is the most specific proteasome inhibitor that acts by covalently binding to an enzymatic subunit (40). Like cbz-LLF-CHO, it predominantly blocks the chymotryptic-like, and tryptic-like activities of proteasomes.

It was recently reported that processing of signal peptides in the ER of T2 cells was inhibited by 250 μM N-Ac-LLnL (6). Presentation of Jaw1(TM$^-$)[NP_{147}] was not significantly inhibited by N-Ac-LLnL (or N-Ac-LLnM) at concentrations of 200 μM (Fig. 6) or 300 μM (data not shown), demonstrating that this protease activity is not required for processing of Jaw1(TM$^-$)[NP_{147}]. Presentation was also unaffected by NH_4Cl (Fig. 6) or leupeptin (data not shown) indicating that endosomal proteases unlikely to be involved in processing. The sensitivity of Jaw1(TM$^-$)/$NP_{147-155}$ to protease inhibitors demonstrates that it and Jaw1(Lum$^-$)[$NP_{147-155}$] are processed in a fundamentally different manner by TAP-deficient cells.

TAP-independent Processing of Jaw1TM$^-$[NP_{147}] Is Not Lymphoid Restricted. Because a homolog of mouse Jaw1 is expressed endogenously by T2 cells, it was plausible that a protein with Jaw1 receptor activity might participate in delivering Jaw1(TM$^-$)[NP_{147}] to the ER of T2 cells. We examined the presentation of Jaw1(TM$^-$)[NP_{147}] in HeLa cells, which do not detectably express Jaw1 (27). To block the function of TAP, cells were coinfected with a rVV-expressing herpes simplex virus ICP47, a potent inhibitor of human TAP function (41). Coexpression of ICP47 blocked presentation of VV determinants to polyclonal VV-specific T_{CD8^+}, and also full-length NP to $NP_{147-155}$-specific T_{CD8^+} (Fig. 7). By contrast, the presentation of Jaw1(Lum$^-$) [$NP_{147-155}$] or Jaw1(TM$^-$)[$NP_{147-155}$] was unaffected. This demonstrates that the TAP-independent presentation of the two protein occurs in cells that do not naturally express Jaw1.

Discussion

We recently found that the liberation of peptides from the COOH terminus of Jaw1 is representative of a process that occurs with both soluble and membrane-bound pro-

teins (Snyder, H.L., manuscript submitted for publication). Based on these findings, we have proposed the C-end rule: antigenic peptides are preferentially produced from the COOH terminus of precursor peptides or proteins in the ER. We believe that this reflects the normal NH_2-terminal trimming of TAP-transported extended peptides with the proper COOH termini for class I binding.

Peptide liberation from the COOH terminus of Jaw occurs much more efficiently than from the other full-length substrates we have tested (an ER-targeted form of NP and CD23). Of these proteins, only Jaw provided sufficient quantities of peptide to detect enhanced expression of K^d in T2 cells (Fig. 4; our unpublished findings). In the case of Jaw1[$NP_{147-155}$], the relatively high efficiency of peptide liberation is presumably associated with the proteolytic processing of its lumenal domain detectable biochemically. The nature of the endoprotease acting on the lumenal domain of Jaw1 is unknown: we presume that an additional aminopeptidase activity is required for final processing of $NP_{147-155}$. Endoprotease activity would appear to be limiting inasmuch as peptide liberation was enhanced by coexpression of yeast Kex2.

Peptides were more efficiently liberated form Jaw1 (Lum^-)[$NP_{147-155}$] than from Jaw1[$NP_{147-155}$]. Jaw1(Lum^-)[$NP_{147-155}$] was produced to take advantage of a potential signal peptidase cleavage site at the junction of the transmembrane and lumenal domains. The efficient liberation of $NP_{147-155}$ from Jaw1(Lum^-)[$NP_{147-155}$] is consistent with the involvement of signal peptidase. Given the mechanism of Jaw insertion into the ER, this would mean that signal peptidase is operating posttranslationally. There is a precedent for this in antigen processing studies. The limited length of ER-targeted peptides dictates that they also must be posttranslationally inserted into the ER (the leader sequence does not emerge from the ribosome before translation is completed), where it is assumed that signal peptidase liberates the peptide from the leader. However, unlike Jaw1, ER-targeted peptides probably enter the ER via the translocon. The efficient processing of Jaw1 suggests that signal peptidase can act on proteins that are inserted into the membrane in a translocon-independent manner.

ER-targeted peptides have been shown to be more immunogenic than full-length proteins (42). This correlates with their highly efficient loading of class I molecules; we recently found that the $NP_{147-155}$ determinant processed from full-length VV-encoded NP is present at ~30 copies/cell while 55,000 copies are recovered from cells expressing the ER-targeted peptide (36). The data in Fig. 6 suggest that Jaw1 is equally or more adept at delivering peptides to class I molecules than simple signal sequences. It will be of interest to determine how Jaw1 compares with signal sequences in enhancing the immunogenicity of defined peptides.

We unexpectedly found that a control Jaw1 construct lacking a transmembrane region was presented in a TAP-independent manner, albeit much less efficiently than ER-targeted Jaw1. We provide immunocytochemical evidence that the protein remains localized to the cytosol. Although we cannot rule out that a minor fraction of the protein is delivered to ER, examination of its sequence reveals no sequences of sufficient hydrophobicity to account for this activity. Most curiously, presentation of $NP_{147-155}$ from its COOH terminus was dependent on a protease activity distinct from the proteasome in being unaffected by lactacystin and blocked by cbz-LL-CHO. The same activity was not required for TAP-independent presentation of the $NP_{147-155}$ peptide liberated from the COOH terminus of other polypeptides directly targeted to the ER. This suggests that the requisite protease is located in the cytosol.

The other major defined cytosolic proteases inhibited by Z-LL-CHO, Z-LLL-CHO, and Z-LLF-CHO are the calpains. However, these are unlikely to be required for the presentation of Jaw1(TM^-)[$NP_{147-155}$], because two additional potent calpain inhibitors failed to affect presentation, even at quite high concentrations. Thus, it appears that we have uncovered a novel cytosolic protease that contributes to antigen processing. Because all of our experiments have used VV to express Jaw1(TM^-)[$NP_{147-155}$], it is possible that the protease is a VV gene product. Similarly, it is possible that transport of the Jaw1(TM^-)[$NP_{147-155}$]-derived peptide into the ER is dependent on an ongoing VV infection.

The putative cytosolic protease may be necessary to maintain the ability of cells to transport the intact Jaw1(TM^-)[$NP_{147-155}$] molecule to the ER. Alternatively, the protease may be involved in producing a fragment from Jaw1(TM^-)[$NP_{147-155}$] that is exported into the ER. This fragment cannot be the naturally processed peptide, because this peptide, when expressed as a minigene product, is not presented by T2 cells under the same conditions. The mechanism by which the fragment gains access to the ER appears to be related to the special properties of the cytosolic domain of Jaw1, because neither $NP_{147-155}$ nor other peptides are presented in a TAP-independent manner when appended to the COOH terminus of cytosolic NP (Snyder, H.L., I. Bačík, J.W. Yewdell, T.W. Behrens, and J.R. Bennink, manuscript submitted for publication). Because TAP-independent presentation of Jaw1(TM^-)[$NP_{147-155}$] is observed in cells that do not express Jaw1, the mechanism of transport is unlikely to be strictly related to the normal function of Jaw1.

There are a number of examples of TAP-independent peptide presentation that cannot be attributed with reasonable certainty to translocon-mediated transport. RMA/S cells, which lack the TAP2 subunit, are able to present peptides from numerous cytosolic proteins, although at much lower efficiency than parental RMA cells (43–47). However, this has been claimed to result from TAP1 partially functioning (48). By contrast, T2 cells do not detectably present cytosolic antigens presented by RMA/S cells, and human TAP1 has an absolute requirement for TAP2 to transport peptides in T2 cells (13). T2 cells have been reported to present cytosolic peptides produced from transfected minigenes (21). In this case, the levels of direct peptide production are enormous compared with the relatively inefficient liberation of peptides from cytosolic proteins by

cellular proteases, and a distinct route into the ER may be used that is less efficient than that used for longer polypeptides. An additional complicating factor in the case of transfectants is the possibility that peptides gain access to the ER due to alterations associated with the process of mitosis, where the nuclear envelope (and the entire secretory pathway) must disassemble and reassemble.

The TAP-independent presentation of Jaw1(TM$^-$)[$NP_{147-155}$] is most consistent with an antigen-processing pathway that depends on a novel cytosolic protease activity and a novel mechanism for translocation into the ER. Our working hypothesis is that a COOH-terminal cleavage product of Jaw1(TM$^-$)[$NP_{147-155}$] is translocated into the ER by a ubiquitously expressed transporter that functions to deliver larger polypeptides to the secretory pathway. It seems likely that this pathway is not a major source of class I-binding peptides, and that its detection was facilitated by exploiting the C-end rule liberation of the peptide once the protein was translocated into the ER. Further, the $NP_{147-155}$-specific T_{CD8^+} used to detect presentation of Jaw1(TM$^-$)[$NP_{147-155}$] are capable of lysing cells expressing <30 K^d-peptide complexes per cell (36). Together, these factors suggest that the primary function of this pathway is nonimmunological in nature.

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Transporter (TAP)-independent processing of a multiple membrane-spanning protein, the Epstein-Barr virus latent membrane protein 2

Antigen presentation to $CD8^+$ cytotoxic T lymphocytes (CTL) usually involves proteolytic cleavage of antigen in the cytosol and the delivery of epitope peptides onto major histocompatibility complex class I molecules in the endoplasmic reticulum (ER) via the heterodimeric peptide transporter TAP1/TAP2. In the few exceptional cases where TAP-independent presentation of an endogenously expressed protein has been observed, the epitope-containing domain of the protein either has naturally accessed or has been directed into the ER lumen where it is thought to become susceptible to ER proteases. Here, we describe a novel example of TAP-independent processing involving the Epstein-Barr virus (EBV) latent membrane protein LMP2, a multiple membrane-spanning protein with minimal projection into the ER. Expression of LMP2 in the TAP^- T2 cell line, whether from the resident EBV genome or from a recombinant vaccinia virus vector vacc-LMP2, rendered the cells sensitive to recognition by CTL clones specific for two HLA-A2.1-restricted peptide epitopes, LMP2 329–337 or 426–434. Vacc-LMP2-mediated sensitization to lysis required expression of the antigen *de novo* in T2 cells and was blocked by brefeldin A. In the same experiments, two other EBV-specific CTL epitopes, one derived from LMP2 but restricted through a different HLA allele (A11), the other restricted through A2.1 but derived from a different viral protein (BMLF1), did not display TAP-independent processing. The results are discussed in relation to the unusual topology of LMP2 in the membrane and the position of the epitope peptides within that structure.

1 Introduction

The principal route whereby endogenously expressed proteins are presented to $CD8^+$ cytotoxic T lymphocytes (CTL) is now known, at least in outline [1, 2]. Proteolytic degradation in the cytosol, probably mediated by proteosomes, generates peptide fragments which can be delivered into the endoplasmic reticulum (ER) and thereby onto MHC class I molecules via the heterodimeric peptide transporter TAP1/TAP2 [3, 4]. Peptides with sufficiently high affinity for MHC molecules are able to form stable MHC class I: peptide complexes [5] and these then move through the Golgi to the cell surface where they are open to engagement by T cell receptors of appropriate specificity within the $CD8^+$ CTL repertoire. Mutant cell lines with deletions of the TAP genes, such as the human cell line 721.174 and its derivative T2, display low steady-state levels of MHC class I molecules and are generally unable to present endogenously expressed antigens to CTL [6]. This phenotype can be largely reversed by TAP gene transfection [7–10], emphasizing the central role which the peptide transporter plays in antigen presentation via the class I pathway.

There are, however, rare examples of endogenously expressed proteins that can be processed and presented in TAP^- cells. In particular, T2 cells are able to present some (but not all) CTL epitopes from the human immunodeficiency virus HIV1 env antigen, a transmembrane protein which during synthesis is naturally translocated into the ER via its N-terminal signal sequence [11, 12]. In this case, env processing is thought to be carried out by ER proteases rather than by the cytosolic proteases which normally supply peptides to the TAP pathway. The identity and substrate specificity of these ER proteases remains to be determined, as does the real extent of their involvement in MHC class I presentation. In this context, several other transmembrane or secreted proteins containing epitope sequences in their luminal domains do not display TAP-independent processing [13–15]. However, the principle that peptide fragments generated by an ER-located protease can load onto nascent MHC class I molecules is clear from work in T2 cells showing that the naturally expressed HLA-A2.1 molecules which do mature in this TAP^- environment preferentially carry peptides generated from the signal sequences of transmembrane or secretory proteins [16, 17]. Indeed engineering a known A2.1-restricted epitope into the signal sequence of a transmembrane protein does lead to epitope-specific CTL recognition when that

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Abbreviations: **BFA:** Brefeldin A **BL:** Burkitt's lymphoma **ER:** Endoplasmic reticulum **LCL:** Lymphoblastoid cell line **LMP:** Latent membrane protein **TAP:** Transporter associated with peptide transport

Key words: Antigen presentation / TAP independence / Epstein-Barr virus / Membrane protein

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protein is expressed from a recombinant vaccinia virus vector in T2 cells [18]. It is also possible to sensitize T2 cells to epitope-specific lysis using minigene constructs to express the minimal epitope tandemly linked to an N-terminal signal sequence [19, 20]; in fact, some minigene-encoded epitope peptides seem to be able to access the ER in T2 cells even without a linked signal sequence [21].

Though derived by cell fusion, the T2 cell line [22] retains the Epstein-Barr virus (EBV)-transformed character of its B lymphoblastoid cell line (LCL) parent 721.174 [23]. As in all LCL transformed *in vitro*, the resident EBV genome expresses six nuclear antigens, EBNA 1, 2, 3A, 3B, 3C and -LP, and two latent membrane proteins, LMP 1 and 2 [24]. Cells with an identical pattern of viral antigen expression are generated *in vivo* during the course of natural EBV infection [25] where they elicit a potent CTL response. The reactivation *in vitro* and cloning of such CTL has allowed the antigenic specificities within the EBV-induced T cell response to be analyzed in some detail. This has shown that a particular subset of latent proteins, namely EBNA3A, 3B and 3C, provide the immunodominant epitopes for CTL responses across a range of different HLA class I alleles [24, 26–28]. However, CTL restricted through the relatively common HLA allele A2.1, though usually a minor component of EBV-specific CTL memory in A2.1$^+$ individuals, regularly map to one of two epitopes within a different target antigen, LMP2 ([29] and Lee et al., manuscript in preparation). This is an unusual multiple membrane-spanning protein made up of cytosolic N- and C-terminal sequences and a central core of 12 tandemly arranged transmembrane domains joined by tight loops which show minimal projection beyond the lipid bilayer [30, 31]. Here, we provide evidence that, in contrast to the virus-coded nuclear antigens, epitopes within the LMP2 protein can be processed and presented to CD8$^+$ CTL by a TAP-independent pathway.

2 Materials and methods

2.1 Cell lines

The T1 and T2 cell lines, kindly provided by Dr. P. Cresswell (Yale University School of Medicine, New Haven, CT), were originally produced by fusing the T leukemic line CEM^R with the TAP$^-$ mutant LCL 721.174 [22, 23]. The T1 hybrid has normal antigen-presenting function and expresses the HLA-A1, A30 and B8 alleles of the CEM^R parent and the HLA A2.1 and B51 alleles of the 721.174 parent; the T2 hybrid was derived from T1 by selecting for loss of the CEM^R HLA locus and resembles its 721.174 parent in being TAP$^-$ and defective in antigen presentation, with a 70–80 % reduction in surface HLA-A2.1 expression compared to T1 and virtually no detectable surface HLA-B51. The T2:B27.05 [13] and T2:A11 [32] lines were produced by stable transfection of T2 with HLA.B27.05 and HLA.A11 gene expression vectors respectively; they were kindly provided by Professor A. McMichael (Institute for Molecular Medicine, Oxford, GB) and Dr. M. Masucci (Karolinska Institute, Stockholm, Sweden). All cell lines were cultured in RPMI 1640 containing 10 % FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (growth medium) supplemented in the case of transfectants with 500 μg/ml G418 as a selection marker.

Fibroblast cultures were established from small skin biopsies and LCL were established by EBV (B95.8 strain) transformation of peripheral blood B cells, in all cases from healthy laboratory donors of known HLA type. The ELI-BL cell line (HLA-A2, A23; B7, B22) was derived from an EBV$^+$ Burkitt's lymphoma (BL) biopsy as described [33]; it retains the biopsy-like group I phenotype with EBV antigen expression limited to EBNA1, shows very low-level TAP expression and has defective antigen presenting function as described for other group I BL lines [34]. The ELI-LMP1 clone (kindly provided by Professor M. Rowe) was obtained by transfecting ELI-BL with a construct stably expressing the EBV latent membrane protein LMP1 [35]; this clone now has restored TAP expression as described in earlier work [34]. The ELI-LCL was derived from normal B cells of the same BL patient by B95.8 EBV transformation.

2.2 CTL clones

EBV-specific CTL clones were derived from healthy laboratory donors or infectious mononucleosis patients of known HLA type by co-culturing PBMC in growth medium with the autologous LCL (irradiated 4000 rad) at a responder-to-stimulator ratio of 40:1. After 7 days, the T cells were fed with growth medium and restimulated with irradiated autologous LCL and, after 14 days, were cloned by limiting dilution at 0.3 or 3 cells per well in 96-well round-bottom tissue culture plates. Each well contained 10^5 mixed allogeneic buffy coat feeder cells (irradiated 4000 rad) and 10^4 irradiated autologous LCL in 100 μl growth medium with 0.5 μg/ml phytohemagglutinin (PHA). After 3 days, growth medium containing 60 % supernatant from the MLA 144 cell line and 200 IU/ml recombinant IL-2 was added (100 μl/well). After a further 7–11 days, growing clones were transferred to 24-well tissue culture plates with each well containing 10^6 mixed irradiated allogeneic buffy coat feeder cells and 10^5 irradiated autologous LCL in 1 ml growth medium with 0.5 μg/ml PHA. After 3 days, growth medium containing 60 % MLA 144 supernatant and 200 IU/ml recombinant IL-2 was again added (1 ml/well). CTL clones were maintained by weekly feeding with growth medium containing 30 % MLA 144 supernatant and recombinant IL-2 (100 IU/ml) and were restimulated every week with irradiated autologous LCL. EBV-specific CTL clones were then identified using a chromium release assay by their ability to lyse targets expressing individual EBV genes from recombinant vaccinia virus vectors (see below). Clones were then tested in peptide sensitization assays to define the target epitope using 14- and 15-mer synthetic peptides (overlapping by 10 amino acids) representing the entire sequence of the relevant EBV protein (see, for example [29]). The CTL clones used in this study were isolated from the following donors: RT (HLA A2.1, 24; B27.05, 35), MR (HLA A2.1, 29; B8, 40), WT (HLA A2.1; B14, 15), KS (HLA A2.1, 11; B35, 40), LY (HLA A1, 24; B27.02, 35), YC (HLA A11, 24; B15, 51) and IM69 (HLA A2.1, 32; B7, B15). Further details on each of these clones are given in Table 1. In some experiments, EBV-specific CTL lines, generated and maintained as described [26] were used as a source of effectors.

Table 1. HLA restriction and epitope specificities of CTL clones

CTL clone[a)]	HLA restriction	EBV target epitope	Target epitope sequence	Cloned at (cells/well)	Reference
RTc30	A2.1	LMP2 426–434	CLGGLLTMV	0.3	[29]
RTc44	A2.1	LMP2 426–434	CLGGLLTMV	0.3	[29]
RTc144	A2.1	LMP2 426–434	CLGGLLTMV	3	[29]
MRc35	A2.1	LMP2 426–434	CLGGLLTMV	3	[29]
MRc41	A2.1	LMP2 426–434	CLGGLLTMV	3	[29]
WTc4	A2.1	LMP2 426–434	CLGGLLTMV	3	[29]
RTc11	A2.1	LMP2 329–337	LLWTLVVLL	3	*
KSc1	A2.1	LMP2 329–337	LLWTLVVLL	0.3	*
RTc5	B27.05	EBNA3C 258–266	RRIYDLIEL	0.3	[36]
LYc26	B27.05[b)]	EBNA3C 258–266	RRIYDLIEL	0.3	[36]
YCc47	A11	LMP2 340–350	SSCSSCPLSKI[c)]	3	*
YCc58	A11	LMP2 340–350	SSCSSCPLSKI[c)]	3	*
IM69c56	A2	BMLF1 280–288	GLCTLVAML	3	**
IM69c62	A2	BMLF1 280–288	GLCTLVAML	3	**

a) Initials identify the donor, numbers identify the individual clone.
b) Clone derived from a B27.02 donor recognizes the epitope in the context of B27.02 and B27.05 [36].
c) Minimal epitope not yet defined.
*) Lee et al., manuscript in preparation.
**) Steven et al., manuscript in preparation; clones kindly provided by Neil Steven and Nicola Annels.

2.3 Cytotoxicity assays

Targets were incubated with $[^{51}Cr]O_4$ for 2 h, washed and incubated with CTL at known effector-to-target ratios in a standard 4 h chromium release assay. Monoclonal antibody (mAb) blocking assays were carried out using the pan-HLA class I-specific mAb W6/32 and control mAb as described [37]. For experiments using recombinant vaccinia, target cells were infected with the virus for 2 h at a multiplicity of infection of 10:1, and then incubated in growth medium for a further 15 h before labeling with chromium. The recombinant vaccinia viruses used in this study have been described previously [26]. In some experiments, the vaccinia virus was inactivated by exposure to ultraviolet light (25 min at a distance of 15 cm from a 30 W bulb) or by heat treatment (10 min at 70 °C); total inactivation of the virus was confirmed by the elimination of plaque-forming ability on a monolayer of TK-143 cells. To study CTL recognition of synthetic peptides, labeled targets were incubated with a known concentration of peptide in 1 ml growth medium for 1 h and then washed. Peptides were synthesized by J. Fox (Alta Bioscience, University of Birmingham, GB) using fluorenylmethoxycarbonyl chemistry, dissolved in DMSO and added to target cells at known dilutions, using an equivalent dilution of peptide-free DMSO as a control [36].

To study the effect of brefeldin A (BFA) on the ability of T2 cells to process and present vaccinia-encoded antigen, cells were incubated with BFA at a concentration of 1 μg/ml throughout the entire experiment (*i.e.* from the moment cells were infected with the vaccinia vector to the end of the chromium-release assay). As a control, mock-infected cells were treated with BFA in the same way, but were incubated with peptide for 1 h prior to use as targets in the assay. The effect of chloroquine was studied by incubating cells in the presence of this drug at a concentration of 500 μM during the 2 h vaccinia infection, and then at a concentration of 50 μM for the remainder of the assay. We have previously shown that this schedule of chloroquine treatment is able to block the processing of exogenous antigen for HLA class II-restricted presentation by LCL cells [38]. To study the possibility that vacc-LMP2-infected cells shed LMP2 peptide fragments at the cell surface, cytotoxicity assays were conducted on chromium-labeled $A2.1^+$ PHA blasts which had been pre-incubated for 1 h with the relevant LMP2 epitope peptides as above, with filtered supernatant from cultures of a vacc LMP2-infected A2.1-positive LCL and the PHA blasts then washed and used as targets, or with equal numbers of cells from a vacc LMP2-infected $A2.1^-$ LCL (unlabeled) and the mixture then added at double the normal target cell input to provide the same number of chromium-labeled PHA blast targets per well as in the rest of the assay.

3 Results

3.1 Recognition of T2 cells by LMP2-specific, HLA-A2.1-restricted CTL

The initial series of experiments involved CTL clones reactivated *in vitro* from EBV-infected $A2.1^+$ donors which recognize the LMP2 epitope CLGGLLTMV (LMP2 426–434) in the context of HLA-A2.1 [29]. We found that individual clones with this specificity differed in the extent to which they recognized $A2.1^+$ LCL tagets; *i.e.* cells expressing LMP2 from the resident EBV genome. Note that such inter-clone variability in LCL recognition is not peculiar to the LMP2 response but is also a feature of CTL reactivated *in vitro* specific for a number of defined epitopes within the EBNA proteins [39]. Using those LMP2-specific clones which did show significant lysis of A2.1-matched LCL, we reproducibly observed that the $A2.1^+$ TAP^- T2 line was also recognized. Representative results from two clones, WT c4 and RT c44, both specific for LMP2 426–434 but derived from different donors, are shown in Fig. 1. These effectors clearly recognized LMP2 when expressed from a recombinant vaccinia virus vector in A2.1-matched fibroblasts. They also recognized an A2.1-matched LCL but not

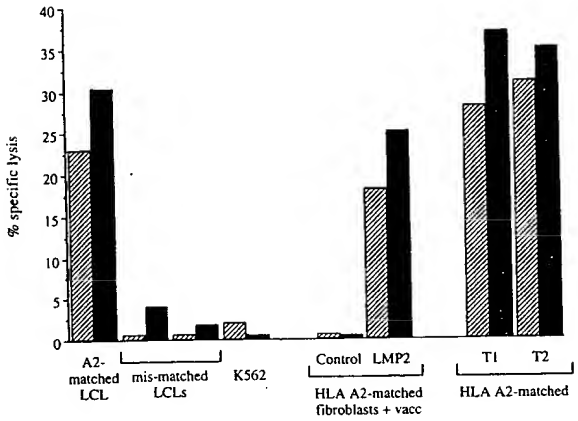

Figure 1. Recognition of the T2 target cell line by HLA A2.1-restricted LMP2-specific effectors. Results of a cytotoxicity assay, expressed as percent specific lysis, are shown for the following targets: allogeneic LCL either HLA A2.1-matched or completely HLA class I-mismatched with the effectors, the NK-sensitive cell line K562, HLA A2.1-matched fibroblasts infected either with a control vaccinia (vacc-TK$^-$) or with vacc-LMP2, and the HLA A2.1-matched T1 and T2 cell lines. Results are shown for two different HLA A2.1-restricted CTL clones, WTc4 (▨) and RTc44 (■), both specific for the LMP2 426–434 epitope and tested at effector:target ratios of 3:1 and 5:1, respectively. Lysis of the A2.1-matched LCL and of T1 and of T2 cells was reduced by 50–60% in the presence of the pan-HLA class I-specific mAb W6/32, but was not affected by a mAb to HLA class II antigens or by isotype-matched control mAb [37].

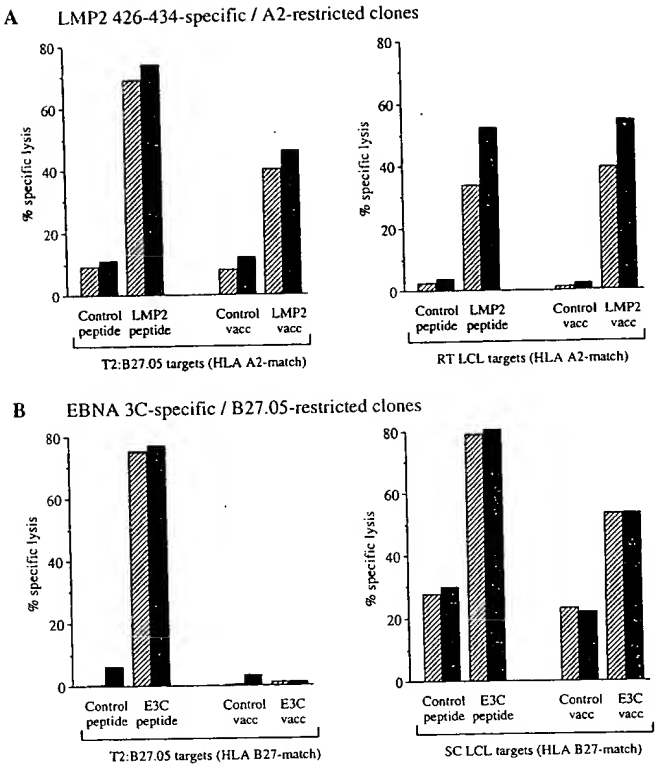

Figure 2. TAP-independent processing of the HLA A2.1-restricted LMP2 426–434 epitope. Results of cytotoxicity assays, expressed as percent specific lysis, with the following effector:target combinations. (A) T2:B27.05 and RT LCL targets (both HLA A2.1-matched with the effector cells) pre-exposed either to the LMP2 426–434 peptide, to a control peptide (EBNA3C 258–266), to vacc-LMP2 or to a control vaccinia (vacc-E3C), then exposed to two different HLA-A2.1-restricted CTL clones, MRc35 (▨) and MRc41 (■), both specific for the LMP2 426–434 epitope and tested at an effector:target ratio of 5:1. In the same experiments, infection with vacc-LMP2B (expressing a truncated form of LMP2 lacking the first 119 amino acids of the N-terminal domain) also sensitized both T2:B27.05 and RT LCL target cells to lysis at 70–80% of the levels shown for vacc-LMP2. (B) T2:B27.05 and SC LCL targets (both HLA B27.05-matched with the effector cells) pre-exposed either to the EBNA3C 258–266 peptide, to a control peptide (LMP2 426–434), to vacc-E3C or to a control vaccinia (vacc-LMP2), then exposed to two different HLA-B27.05-restricted CTL clones, RTc5 (▨) and LYc26 (■), both specific for the EBNA3C 258–266 epitope and tested at an effector:target ratio of 5:1. All peptides were used at a final concentration of 0.2 μg/ml.

mismatched LCL or the NK-sensitive target K562; however, they did mediate significant lysis of T2 cells as well as of T1 (the A2.1$^+$, TAP$^+$ hybrid from which T2 was derived). This was not nonspecific lysis, since it could be blocked by a mAb against HLA class I molecules but not by control antibodies (Fig. 1, legend). Furthermore, killing of T2 was only observed with LMP2-specific A2.1-restricted CTL clones and not with several other clones recognizing EBNA-derived epitopes in the context of other HLA class I alleles (for example, see Fig. 2B).

3.2 T2 cells can present vaccinia-expressed LMP2

Further assays were then carried out using clones also specific for the LMP2 426–434 epitope in the context of A2.1, but with much lower base-line reactivity against A2.1-matched LCL and T2 targets, thereby allowing these targets to be tested for their ability to present LMP2 endogenously expressed at high levels from a recombinant vaccinia virus vector (vacc-LMP2). In these particular experiments, we sought to compare LMP2 processing with that of a different EBV target protein, the nuclear antigen EBNA3C, and therefore used T2 cells stably transfected to express HLA B27.05, a restriction element for EBNA3C-specific CTL [36]. Fig. 2A presents the results obtained using two different A2.1-restricted clones, MRc35 and MRc41, specific for LMP2 426–434. The T2:B27.05 cells were clearly sensitized to specific lysis not just by exogenous loading of the relevant LMP2 epitope peptide, but also by infection with the vacc-LMP2 recombinant; such results were essentially similar to those seen when these same effectors were tested on peptide-loaded or vacc-LMP2-infected cells of a standard A2.1$^+$ LCL, RT. These findings were in marked contrast to the results obtained in the same experiment using two B27.05-restricted clones, RTc5 and LYc26, specific for the EBNA3C 258–266 epitope. Here, T2:B27.05 cells were clearly recognized after exogenous loading of the peptide but not after vacc-EBNA3C infection (Fig. 2B). Note that these EBNA3C-specific CTL clones did show some significant lysis of the standard B27.05-matched LCL, SC, by virtue of EBNA3C expression from its resident EBV genome, but this was clearly increased both by exogenous peptide loading and by vacc-EBNA3C infection.

The results shown in Fig. 2A, were subsequently confirmed on up to ten further occasions using a variety of different LMP2 426–434 epitope-specific clones from A2.1$^+$

donors. In addition, we repeated the assays using a different vaccinia recombinant encoding LMP2B, a truncated form of LMP2 which is also naturally expressed in LCL cells and which lacks the first 119 amino acids of the full-length protein [30]; vacc-LMP2B infection again reproducibly sensitized T2 cells to recognition by LMP2 426–434-specific CTL (see Fig. 2, legend). These experiments all included as control effectors either EBNA3C-specific, B27.05-restricted clones tested on T2:B27.05 targets as in Fig. 2B, or EBNA3B-specific, A11-restricted clones [32] tested on T2:A11 (data not shown). In each case, a pattern of results similar to that shown in Fig. 2B was obtained, confirming that the presentation of EBNA proteins for CTL recognition is indeed prohibited in T2 cells.

3.3 LMP2 presentation requires endogenous expression of the protein in T2 cells

We were concerned to check that in the above experiments the recombinant vaccinia preparation itself was not acting as a source of pre-formed LMP2 antigen or LMP2 peptide fragments which might access HLA-A2.1 molecules either intracellularly by some novel TAP-independent route [40] or by direct binding at the target cell surface. A number of experiments were therefore carried out, first involving the use of vaccinia virus preparations which had been rendered noninfectious by heating or by UV irradiation. Representative data are shown in Fig. 3A. As before, the LMP2-specific effectors recognized T2 cells pre-loaded with the synthetic epitope peptide (with or without heat treatment of the peptide) and following active infection of the target cells with vacc-LMP2; however, neither of the inactivated virus preparations sensitized T2 cells to lysis. Furthermore, BFA, a drug which blocks HLA class I exocytosis beyond the *cis*-Golgi compartment and therefore prevents the surface presentation of HLA class I-peptide complexes formed in the ER [41, 42], completely abrogated the vacc-LMP2-mediated sensitization of T2 cells to CTL recognition if added at the time of infection, whereas it did not affect sensitization by exogenous peptide (Fig. 3B). In similar experiments, chloroquine, a drug which blocks antigen processing by endosomal/lysomal pathways [43], had no significant effect on the recognition of vacc-LMP2-infected cells (data not shown). Further experiments examined the possibility that the LMP2 synthesized in vaccinia-infected cells was being broken down at the plasma membrane to generate antigenic fragments which could bind back to surface A2.1 molecules, thereby sensitizing the cells to CTL recognition. In fact, we found no evidence for such a mechanism, since A2.1$^+$ PHA blast targets, which were clearly sensitized to CTL by pre-exposure to exogenous LMP2 peptide, were not killed when pre-exposed to the supernatant of vacc-LMP2-infected LCL cells (data not shown) or when co-cultivated with such cells before and during the CTL assay (Fig. 3C).

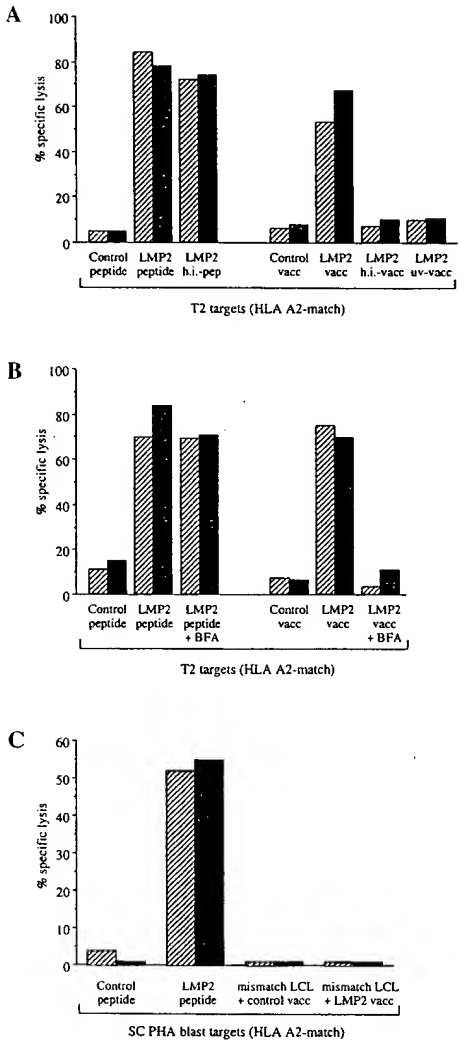

Figure 3. Requirements for vacc-LMP2-mediated presentation of the LMP2 426–434 epitope in T2 cells. Results of cytotoxicity assays, expressed as percent specific lysis, involving the following targets. (A) T2 target cells pre-exposed to a control peptide (EBNA3C 258–266), to the LMP2 426–434 peptide untreated or heat-inactivated at 70 °C (h.i.), to a control vaccinia (vacc-E3C), or to vacc-LMP2 either untreated or heat-inactivated at 70 °C (h.i.) or UV-inactivated (uv-vacc). (B) T2 target cells pre-exposed to a control peptide (EBNA3C 258–266), to the LMP2 426–434 peptide, to a control vaccinia (vacc-TK$^-$) or to vacc-LMP2 as above, but where some targets had been maintained in BFA from the time of vacc infection (see Sect. 2.3). (C) HLA-A2.1$^+$ SC PHA blast targets pre-exposed to a control peptide (EBNA3C 258–266) or to the LMP2 426–434 peptide as above, or SC PHA blast targets mixed with an equal number of unlabeled cells from a mismatch LCL (HLA A2.1$^-$) that had been pre-exposed to a control vaccinia or to vacc-LMP2 as above. In each of the above assays, the effector cells were HLA A2.1-restricted CTL clones specific for the LMP2 426–434 epitope and tested at an effector: target ratio of 5:1. The CTL clones were (A) RTc144 (▨) and RTc30 (■), (B) MRc41 (▨) and RTc30 (■), and (C) RTc144 (▨) and MRc41 (■).

3.4 T2 assays involving other LMP2-derived or A2.1-restricted epitopes

The experiments using recombinant vaccinia-infected T2 cells as targets for CTL recognition were then extended to include a series of other CD8$^+$ clones with potentially informative reactivities. These were clones from donors RT and KS which are also A2.1-restricted and LMP2-specific, but recognize a different peptide epitope LLWTLVVLL (LMP2 329–337), clones from donor IM69 which are also A2.1-restricted but recognize a defined peptide epitope GLCTLVAML within the EBV immediate early lytic cycle protein BMLF1, and clones from donor

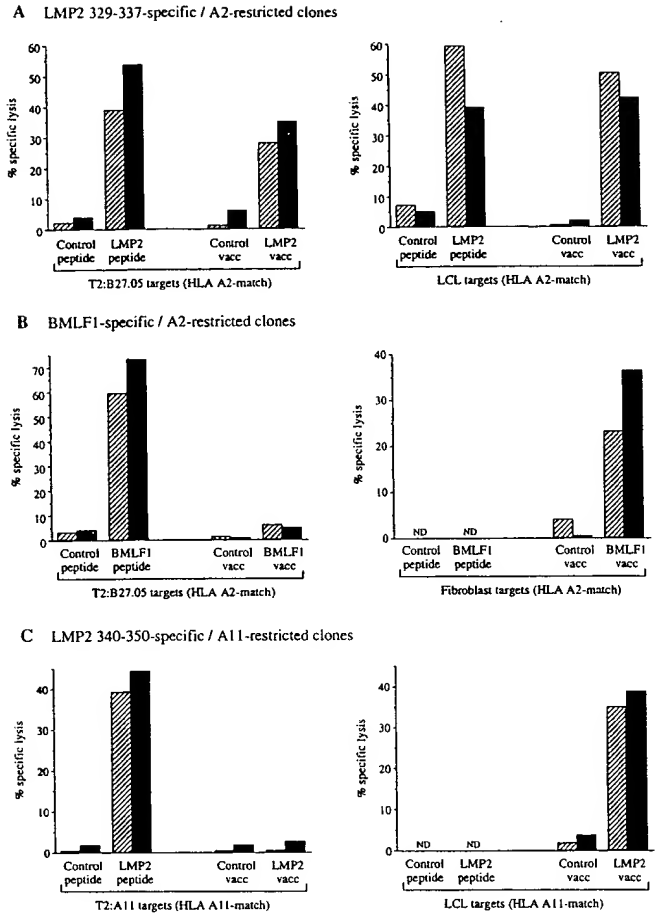

Figure 4. TAP-independent processing of a second HLA A2.1-restricted LMP2 epitope (LMP2 329–337) but not of an A2.1-restricted epitope in BMLF1 or of an A11-restricted epitope in LMP2. Results of cytotoxicity assays, expressed as percent specific lysis, are shown for the following effector:target combinations. (A) T2:B27.05 and a standard HLA-A2.1$^+$ LCL pre-exposed either to the LMP2 329–337 peptide, to a control peptide (EBNA3C 258–266), to vacc-LMP2 or to a control vaccinia (vacc-E3C), then exposed to two different HLA A2.1-restricted CTL clones, KSc1 (▨) and RTc11 (■), both specific for the LMP2 329–337 epitope and tested at an effector:target ratio of 5:1. (B) T2:B27.05 and HLA A2.1$^+$ fibroblasts pre-exposed to the BMLF1 280–288 peptide, to a control peptide (LMP2 426–434), to vacc-BMLF1 or to a control vaccinia (vacc-TK$^-$), then exposed to two different HLA A2.1-restricted CTL clones, IM69c56 (▨) and IM69c62 (■), both specific for the BMLF1 280–288 epitope and tested at an effector:target ratio of 10:1 and 5:1, respectively. (C) T2:A11 and a standard HLA A11$^+$ LCL pre-exposed either to the LMP2 340–350 peptide, to a control peptide (EBNA3B 416–424), to vacc-LMP2 or to a control vaccinia (vacc-TK$^-$), then exposed to two different HLA A11-restricted CTL clones, YCc58 (▨) and YCc47 (■), both specific for the LMP2 340–350 epitope and tested at an effector:target ratio of 5:1. N.D.: not done.

YC which are specific for another LMP2 epitope SSCSSCPLSKI (LMP2 340–350), but presented in the context of a different HLA allele, A11 (see Table 1).

The reactivities of these clones in T2 assays are illustrated by representative data shown in Fig. 4. We found that the A2.1-restricted LMP2 329–337 epitope was consistently presented in vacc-LMP2-infected T2 cells, the pattern of results (Fig. 4A) being very similar to that observed in earlier experiments with A2.1-restricted clones recognizing the LMP2 426–434 epitope. However, not all A2.1-restricted epitopes were presented in T2. Thus the BMLF1-specific clones could lyse T2 cells when exogenously loaded with the relevant synthetic peptide but not when infected with a BMLF1-expressing vaccinia recombinant; by contrast, infection with the vacc-BMLF1 recombinant could sensitize TAP$^+$ targets (*i.e.* A2.1-matched normal fibroblasts) to recognition by the same effectors (Fig. 4B). Furthermore, not all CTL epitopes within LMP2 displayed a TAP-independent phenotype. Thus A11-restricted CTL specific for the LMP2 340–350 epitope reproducibly failed to recognize vacc-LMP2-infected T2:A11 cells, even though they did kill T2:A11 cells pre-coated with the relevant peptide and also vacc-LMP2-infected cells of a standard A11-matched LCL (Fig. 4C).

3.5 A2.1-restricted presentation of LMP2 in a TAP-deficient BL cell background

In previous work, we and others have shown that cell lines derived from BL and retaining the group I phenotype of the original tumor show greatly reduced expression of the TAP1/TAP2 heterodimer when compared to LCL and are generally unable to present endogenously expressed EBNA proteins to CTL of appropriate specificity [34, 44]. Interestingly, TAP1/TAP2 expression and to some extent antigen-presenting function can be restored by stable vector-mediated expression of the major EBV growth transforming protein LMP1 [34]. In a final series of experiments, therefore, we sought to test one such group I BL line, the A2.1$^+$ ELI-BL, for its capacity to present the

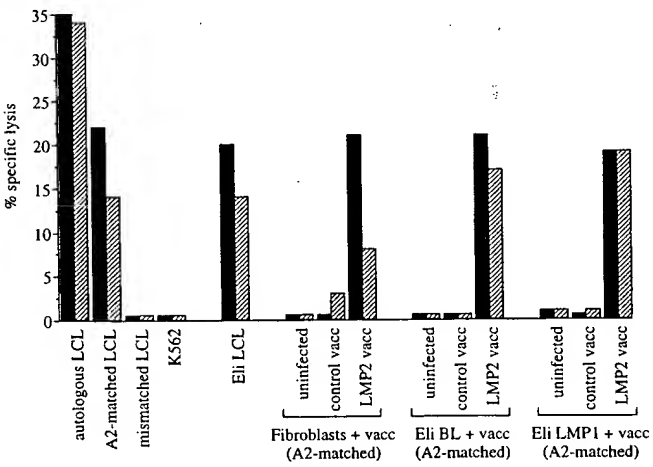

Figure 5. Processing of the HLA A2.1-restricted LMP2 426–434 epitope in the TAP-deficient ELI-BL cell line. Results of a cytotoxicity assay, expressed as percent specific lysis, are shown for the following targets: the autologous LCL, allogeneic LCL either HLA-A2.1-matched or completely HLA class I-mismatched with the effectors, the NK-sensitive cell line K562, the HLA A2.1-matched ELI LCL plus the following HLA A2.1-matched targets either uninfected, infected with a control vaccinia (vacc-TK$^-$) or with vacc-LMP2: normal fibroblasts, ELI-BL and an LMP1$^+$ transfectant of ELI-BL, ELI-LMP1. Effector cells in this assay were a polyclonal CTL line from donor WT with a dominant component of HLA A2.1-restricted LMP2 426–434-specific cytotoxicity [29] and tested at effector:target ratios of 10:1 (■) and 5:1 (▨). Similar results were obtained with CTL clones specific for the LMP2 426–434 epitope (data not shown).

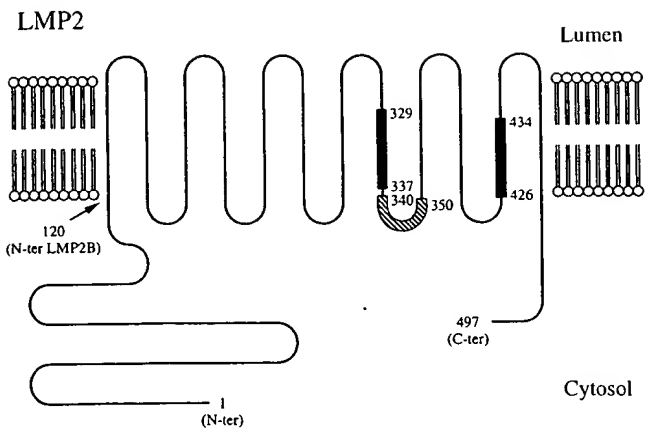

Figure 6. Diagrammatic representation of the topology of the 497-amino-acid LMP2 protein as it is thought to insert in the ER membrane, showing the extent of luminal and cytosolic projections [30, 31]. The presumed positions within this structure of the two A2.1 epitopes LMP2 329–337 and 426–434 (dark bars) and of the A11 epitope LMP2 340–350 (hatched bar) within this structure are as shown. The truncated form of LMP2, LMP2B, initiates at a methionine residue which is at +120 in the full-length LMP2 protein (arrow).

LMP2 426–434 epitope to A2.1-restricted CTL. Included as a positive control in these experiments was an LMP1-transfectant of ELI-BL, ELI-LMP1 [35], in which TAP levels had been restored. Fig. 5 presents the results seen in one such experiment, in this case using as effectors a CTL line from donor WT with a dominant A2.1-restricted LMP2-specific component [29]. We found that the parental ELI-BL cells were as efficient as normal fibroblasts or as the LMP1-transfected ELI clone at presenting vaccinia-expressed LMP2 for A2.1-restricted CTL recognition. Similar results were obtained using individual LMP2 426–434 epitope-specific CTL clones (data not shown).

4 Discussion

We initially observed that those A2.1-restricted LMP2 426–434-specific CTL clones which were active against A2.1-matched TAP-positive LCL lysed the T2 target line equally well (Fig. 1). This was quite unexpected, because CTL clones specific for defined epitopes in the immunodominant EBNA3 proteins and restricted through other HLA alleles [26, 32, 36] had never shown either specific recognition of a T2 transfectant expressing the relevant HLA restricting allele or anomalous killing of T2 itself (see for example Fig. 2B). An unusual feature of the EBV system, true for EBNA- as well as for LMP-specific CTL, is that many clones lyse the natural LCL target rather poorly if at all *in vitro* but mediate strong lysis of the same cells pre-exposed to the cognate peptide or infected with a vaccinia virus expressing the cognate antigen [39]. We attribute this interclonal variability to the fact that some clones require a relatively high density of HLA/peptide complexes on the target cell surface to achieve recognition and lysis. In this context, some of the LMP2 426–434-specific clones which were not active against standard $A2.1^+$ LCL nevertheless consistently showed low but apparently significant levels of killing (typically 10–15 % specific lysis) of the T2 line or of T2 transfectants (see Fig. 2A). This might be expected if the LMP2 426–434 epitope, accessing the ER by a TAP-independent route, is able to compete for nascent A2.1 molecules more effectively in the T2 line than in a standard LCL where there is an efficient supply of peptides via the TAP pathway. The evidence for TAP-independent processing of LMP2 was strengthened considerably by the results of experiments using a recombinant vaccinia vector to enhance expression of the antigen in target cells. This allowed us to assay a wider range of LMP 426–434-specific A2.1-restricted clones, all of which clearly showed lysis of vacc-LMP2-infected T2 cells (Fig. 2A, 3) at levels equivalent to those seen for vacc-LMP2-infected cells of a standard $A2.1^+$ LCL. Parallel assays with control clones, for instance EBNA3C-specific B27.05-restricted CTL, never showed recognition of T2-derived targets despite the presence of the relevant restricting allele and vaccinia-mediated expression of the cognate target antigen (Fig. 2B).

In these experiments, we were concerned that the vacc-LMP2 virus preparation might itself be a source either of the pre-formed antigenic peptide, which could thus bind to surface A2.1 molecules directly, or of LMP2 fragments which could be processed for A2.1-restricted presentation by a TAP-independent route. It is clear that in some cell types, exogenously supplied antigens can access the MHC class I presentation pathway, especially if the antigen is delivered in particulate form (reviewed [45]). Interestingly, where this phenomenon has been observed in LCL cells, it appears to involve a novel pathway which is both TAP-independent and BFA-resistant [46–48], suggesting the loading of processed peptides onto class I molecules in a post-Golgi compartment. By contrast, in the present work, vacc-LMP2-mediated sensitization of T2 cells to LMP2-specific effectors clearly required endogenous expression of the antigen, since both heat-inactivated and UV-inactivated virus preparations did not mediate cytolysis (Fig. 3A). Moreover, the inhibition of LMP2 presentation by BFA (Fig. 3B) was also consistent with a processing pathway which involved peptide loading onto class I molecules in the ER. However, these experiments left open the possibility that LMP2 was actually being degraded by cell or serum proteases at the surface of, or after release from, infected cells and that epitope peptides generated in this way could then bind to surface A2.1 molecules; the observed inhibition by BFA would then simply reflect the drug's ability to block the export of newly synthesized LMP2 to the plasma membrane. In fact, this was clearly not the case since neither vacc-LMP2-infected cells themselves nor supernatants from such cells were able to sensitize $A2.1^+$ targets to LMP2-specific effectors (Fig. 3C).

The identification of two new CTL epitopes in LMP2 (one restricted through A2.1, the other through A11) and of an A2.1-restricted epitope in an early cycle EBV protein, BMLF1, allowed us to examine the generality of the results observed using LMP2 426–434-specific effectors. Interestingly, we found that the second A2.1-restricted epitope in LMP2, 329–337, was also processed and presented in TAP^- cells, whereas the adjacent A11-restricted epitope in the LMP2 sequence, 340–350, was not (Fig. 4A, C). This could not be explained by inadequate level of A11 expression in the T2:A11 transfectant since exogenous addition of the LMP2 340–350 peptide to these cells medi-

ated strong lysis (Fig. 4C; see also [32]). The hydrophobic nature of the two A2.1 epitopes in LMP2 (CLGGLLTMV, LLWTLVVLL) compared to the much less hydrophobic A11 epitope (SSCSSCPLSKI) suggests one possible explanation for the present data. This envisage the cytosolic processing of a small fraction of nascent LMP2 molecules that for some reason fail to insert into the membrane; entry of the derived peptides into the ER will be blocked in a TAP^- cell, except perhaps for very hydrophobic peptides (such as the A2.1 epitopes) which may, like some minigene-encoded epitope sequences [21], gain access by a TAP-independent route. While formally possible, we consider this explanation unlikely because a hydrophobic A2.1 epitope GLCTLVAML in the EBV-coded BMLF1 protein did not show the same TAP-independent phenotype (Fig. 4B). Likewise, others have found that the hydrophobic A2.1 epitope GILGFVFTL within the influenza matrix protein is clearly not presented from the endogenously expressed protein in T2 cells [6, 10] even though the peptide itself may access A2.1 molecules if expressed in these cells from a minigene [21].

We suggest that the unusual TAP-independent processing of the LMP2 329–337 and 426–434 epitopes may be related not to their hydrophobic character *per se*, but to the fact that they are naturally located in the transmembrane domains of a multiple membrane-spanning protein. Fig. 6, illustrates diagrammatically the presumed topology of LMP2 in the lipid bilayer [30, 31]; 12 membrane-spanning domains are flanked by N and C termini whose cytosolic orientation has been confirmed by biochemical studies showing that the N-terminal region physically interacts through a YXXL motif with cytosolic src-family kinases [49, 50]. In this native configuration of LMP2, both A2.1 epitopes are predicted to lie within the lipid bilayer, whereas the A11 epitope is part of a short cytosolic loop between two transmembrane stretches. From what is known of the assembly of membrane proteins, it seems likely that all 12 transmembrane domains are inserted sequentially into the membrane during LMP2 biosynthesis and that only the short intraluminal loops ever make contact with the ER environment ([51, 52], E. Hartmann, personal communication). This clearly distinguishes the present examples of TAP-independent processing, where the relevant epitopes lie within regions of the LMP2 protein not normally exposed to ER proteases, from the examples observed with HIV env [11, 12], where the relevant epitopes are all situated within the env protein's luminal domain. The mechanism of LMP2 processing remains unknown, but in this context it is interesting that two other multiple membrane-spanning proteins, the cystic fibrosis transmembrane conductance regular and Sec61 (a component of the ER membrane's protein translocation apparatus), are now thought to be degraded in the ER membrane by a process involving ubiquitination and proteosome cleavage of their cytosolic domains [53–55]. Indeed, the analysis of Sec61 breakdown in yeast suggests a model in which initial cleavages on the cytosolic face of the protein generate unstable intermediates which are then cleaved further on their luminal face by ER peptidases, perhaps by the signal peptidase itself [55]. If a fraction of membrane-bound LMP2 is similarly degraded, then transmembrane stretches of the protein (containing the A2.1 epitope sequences) might be released and, just as happens to signal sequences released within the lipid bilayer [51, 52], these peptide fragments could then enter the ER and access nascent class I molecules in a TAP-independent manner [16–18]. As more CTL epitopes are identified in the LMP2 sequence, we should be able to determine more clearly how the position of the epitope within the native molecule might influence its ability to be processed in T2 cells.

Finally we draw attention to the fact that the TAP-independent processing of LMP2 is not in any way peculiar to the T2 line, but was also observed in ELI-BL cells (Fig. 5), one of several group I Burkitt tumor-derived lines in which we and others have shown that TAP expression is down-regulated and that conventional antigen processing via the TAP pathway is blocked [34, 44]. Whilst LMP2 is not normally expressed in BL and therefore does not constitute a potential target for CTL-based immunotherapy in that tumor, the situation is different in two other EBV-associated malignancies, nasopharyngeal carcinoma and Hodgkin's disease [24]. These tumors do appear to be $LMP2^+$ and, given the frequency of the A2.1 allele in human populations, a substantial number of these tumors could possibly be targeted by A2.1-restricted LMP2-specific effectors of the kind described here [29]. It is not yet known whether nasopharyngeal carcinoma and Hodgkin's cells will display the same impairment of TAP-dependent processing as displayed by BL; however, this must now be less of a concern since, as the present paper shows, the relevant A2.1 epitopes can be presented even in TAP-deficient cells.

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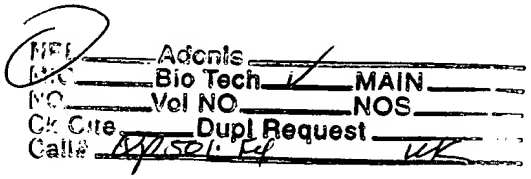

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Minireview

The transporter associated with antigen processing TAP: structure and function

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Abstract The transport of antigenic peptides from the cytosol to the lumen of the endoplasmic reticulum (ER) is an essential process for presentation to cytotoxic T-lymphocytes. The transporter associated with antigen processing (TAP) is responsible for the intracellular translocation of peptides across the membrane of the ER. Efficient assembly of MHC-peptide complex requires the formation of a macromolecular transport and chaperone complex composed of TAP, tapasin and MHC class I molecules. Therefore, structure and function of TAP is important for the understanding of the immune surveillance.

Key words: ABC transporter; Antigen presentation; Transport mechanism; Virus persistence

1. Introduction

Presentation of antigenic peptides by MHC class I molecules to cytotoxic T-lymphocytes (CTL) is an essential process for the cellular immune recognition. These peptides are primarily generated by the proteasome complex, a multisubunit, multicatalytic protease (for review see [1]), and are translocated from the cytosol into the lumen of the endoplasmic reticulum (ER) by a so-called transporter associated with antigen processing (TAP). In the ER, the peptides are loaded onto the newly synthesized MHC class I molecules. Binding of the peptide stabilizes the complex and induces the export to the cell surface for presentation to T cell receptors (Fig. 1A). The class I heterotrimer consists of an MHC encoded heavy chain, β2-microglobulin and peptide and its assembly is well studied (for review see [2,3]). However, less is known of how the peptides are transported into the ER. Some open questions still remain: The amount and quality of peptides generated by the proteasome, the mechanism of the transport as well as the involvement of other factors such as additional chaperones, proteases or transporters. The availability of peptides to the MHC class I molecules could be rate-limiting for antigen presentation, and selectivity of this step could be imprinted onto the pool of dominant and subdominant epitopes. In this review, the current knowledge about the mechanisms underlying the transport of the peptides from cytosol to the lumen of the ER is described.

2. Genomic organization of TAP

Studies concerning various cell lines with a strongly reduced level of MHC class I molecules on the cell surface, strengthened the importance of peptide transport into the ER lumen [4]. Peptides exogenously added or introduced into the ER by signal sequences were efficiently presented, but these cell lines were unable to present intracellular antigens on the cell surface. It soon became clear that this defect was due to deletions in the region of the MHC locus and most likely involved a gene or genes, that were responsible for delivering peptides to the lumen of the ER and/or loading newly synthesized class I molecules with them. In the following, four groups independently described candidate genes for a factor that would transport peptides across the ER membrane [5–8]. In 1991, a WHO nomenclature committee for factors in the HLA system renamed these genes TAP1 (for RING4, PSF1, mtp1 and HAM1) and TAP2 (for RING11, PSF2, mtp2 and HAM2) [9]. Alignment between the sequences of TAP1 and TAP2 showed the highest homology in a stretch of 200 amino acids located at the C-terminus. This region contains three characteristic motifs. The Walker A and B motifs form a highly conserved ATP-binding cassette. A so-called C-loop, which consists of six to eight conserved amino acids, is located in between the Walker A and B motifs. Therefore, the proteins belong to the superfamily of ATP-binding cassette (ABC) transporters. A conserved nucleotide-binding domain (NBD) and a transmembrane domain (TMD) of about six membrane-spanning segments characterize this protein family. A functional unit comprises two NBDs and two TMDs. Functional impact of TAP1 and TAP2 was proven by transfection of defective cell lines with TAP2 and/or TAP1 genes, restoring antigen presenting activity [10,11].

The genomic structures of the human TAP genes have been established [12]. Each gene is located in the MHC II locus of chromosome 6, comprises about 10 kb and consists of 11 exons (Fig. 1B) [13]. Eight of these exons are of the same length, although the intron sizes vary significantly. The remaining three exons 1, 9 and 11 differ in length by 100, 3 and 78 nucleotides, respectively. All 11 intron/exon boundaries are identical in their classes and follow the GT/AG rule. Sequence comparisons from human to salmon TAP1 showed the expected phylogenetic differences (for example 98.8% homology of human with gorilla TAP1, 69.2% with hamster and

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Abbreviations: ABC, ATP-binding cassette; CTL, cytotoxic T-lymphocytes; ER, endoplasmic reticulum; MHC, major histocompatibility complex; NBD, nucleotide-binding domain; TAP, transporter associated with antigen processing; TMD, transmembrane domain

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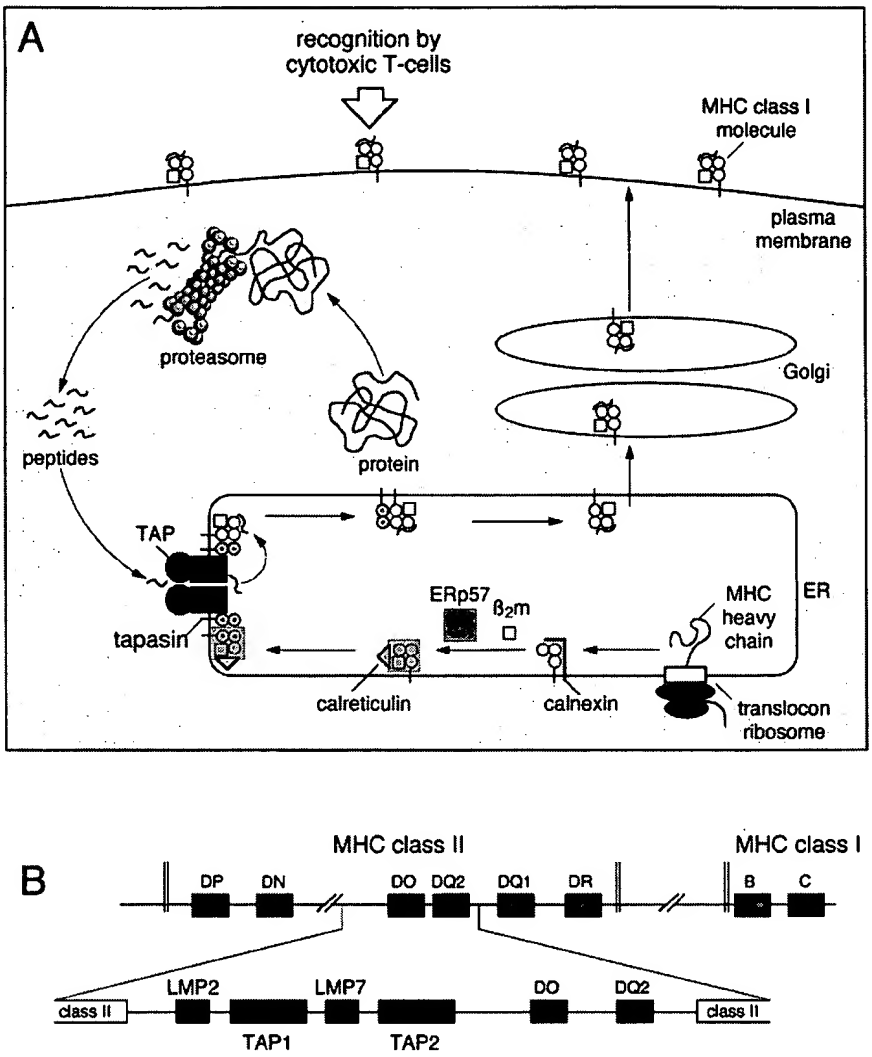

Fig. 1. A: Antigen processing and presentation via MHC class I molecules. Endogenous proteins are degraded by the proteasome. Peptides are transported into the lumen of the ER by the TAP complex. Several molecules are involved in assembly and loading of the MHC class I molecules, including calnexin, calreticulin, tapasin and ERp57. Stable MHC-peptide complexes leave the ER via the Golgi compartment to the cell surface for recognition by cytotoxic T-lymphocytes. Open circles represent folded immunoglobulin domains. B: The location of the TAP genes in the human MHC class II complex (HLA). The scale is intended as a rough guide only.

40% with salmon). The analysis indicates that the degree of relatedness between human TAPs is similar to that between each gene and its homologues in rodents [14]. Although human TAP1 and TAP2 exhibit only 64% homology at the protein level, they have a similar predicted membrane topology. Therefore, it is speculated that the genes have evolved from a common ancestral gene by duplication prior to the development of the adaptive immune system in vertebrates [15].

3. Regulation of TAP genes

MHC class I molecules are expressed at low levels in most cells and are strongly induced by cytokines such as interferons (for review see [16]). Increased expression of MHC class I molecules correlates with increased CTL function. Therefore, it was of special interest to study the gene expression of TAP1 and TAP2. Both TAPs are up-regulated by interferon-γ about 10-fold within 24 h, accompanied by an increased peptide transport capacity. The TAP genes contain no TATA box motifs in the 5'-flanking sequences, but putative GC-rich elements (Sp1-binding sites, 128 nucleotides upstream the translation start codon for TAP1 and 79 nucleotides upstream for TAP2) [12]. Site directed mutagenesis of the Sp1-binding site leads to a 3-fold reduction of basal promoter activity of TAP1 [17]. It should be noted that the TAP1 gene is coordinately regulated by a bidirectional promoter of 593 bp, also directing the divergently transcribed gene of low molecular mass polypeptide 2 (LMP2), a β-type proteasomal subunit, β_1. Both genes are induced by TNF-α via an NF-κB element, which is also found in the class I response element [18]. The cytokine induced expression of TAP1 and LMP2 concordantly with class I genes suggest a mechanism to link transporter levels with class I production.

4. Structural organization of the TAP complex

As mentioned before, human TAP1 (748 amino acids) and

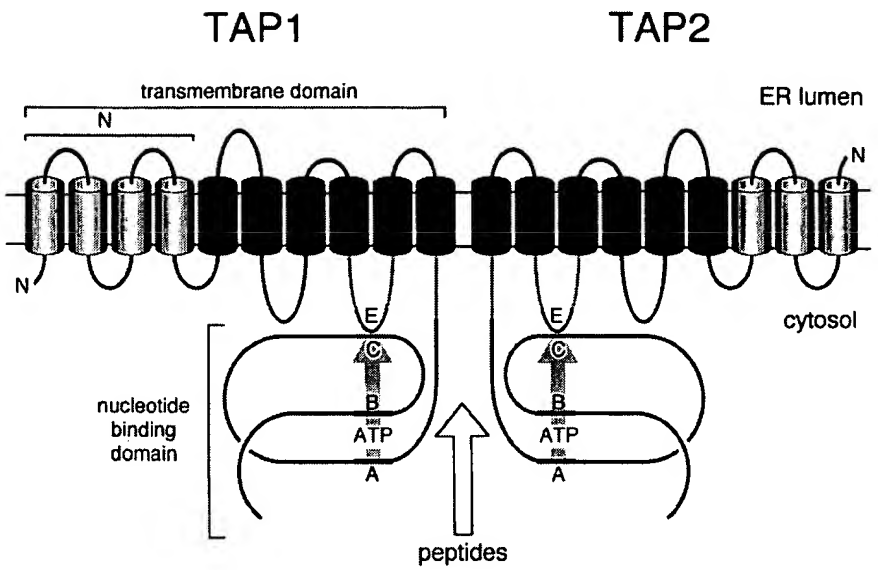

Fig. 2. Membrane topology of the human TAP complex. The transmembrane helices are predicted from hydrophobicity plots and sequence alignments with other ABC transporters. The N-terminal domain (N) of TAP1 and TAP2 is very hydrophobic, comprising three or four predicted transmembrane helices (gray symbols). The nucleotide-binding domain consists of the Walker A and B motifs (A, B) and the so-called C-loop (C), interacting with a 'EAA'-like motif (E) in the last cytosolic loop of the membrane-spanning domains. The orange lines illustrate binding regions for peptides as identified by photo-crosslinking experiments.

human TAP2 (686 amino acids) belong to the superfamily of ABC transporters, comprising a large number of polytopic membrane proteins transporting a diverse set of molecules across membranes in an ATP-dependent manner (for review see [19,20]). The homology with other members of this family points to a functional TAP protein either as a homodimer of TAP1 or TAP2 or a heterodimer. Indeed, immunoprecipitations with antisera directed against TAP1 coprecipitated TAP1 and TAP2 proteins [21]. Comparisons of the phenotypes of different mutant cell lines and heterologous expression in insect cells and yeast suggest that neither TAP1 nor TAP2 can form a functional homodimer, indicating that only a heterodimer is functional [22,23]. Hydrophobicity analysis and sequence alignments with related ABC transporters point to a 2×6 transmembrane helix model of TAP, extended by additional four and three transmembrane helices predicted for the highly diverse N-terminal domain (Fig. 2) [24]. The hydrophobic transmembrane domains are linked to the nucleotide-binding domains, which contain the conserved Walker A and B motifs for ATP binding and hydrolysis and, based on this

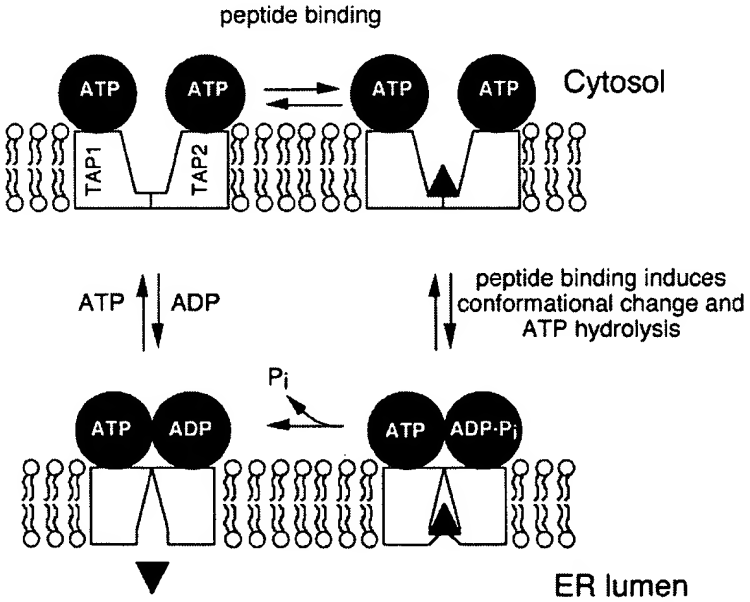

Fig. 3. Model of peptide translocation by the TAP complex. Peptide (dark blue triangles) and ATP/ADP independently bind to TAP in the cytosol. It is unknown, if both NBDs are loaded with ATP. Peptide binding induces a structural reorganization of the TAP complex, triggering ATP hydrolysis and subsequently translocation of the peptide.

model, are located in the cytosol. The C-loop interacts with an 'EAA'-like motif (E) found in the last cytosolic loop of the membrane-spanning domains [25]. The peptide-binding region was mapped by photo-crosslinking of peptides to TAP, digestion by trypsin and/or bromocyan and subsequent immunoprecipitation with antibodies directed against different epitopes of TAP [26]. The analysis of photo-crosslinked fragments revealed a similar binding region for TAP1 and TAP2, comprising the cytosolic loops between TM4 and TM5 and a carboxy-terminal stretch of about 15 amino acids following TM6. So far, the peptide translocation pathway is not known, but it can be speculated that TM5 and TM6 are parts of a translocation channel.

5. Transport mechanism of TAP

Peptide transport by TAP is a multi-step process [27] (Fig. 3). In a fast bimolecular association step, the peptide binds to TAP in an ATP-independent manner [28], followed by a slow isomerization of the TAP complex [29]. It is suggested that this structural reorganization of the molecule triggers ATP hydrolysis and peptide translocation across the membrane. These binding steps primarily determine the selectivity of TAP. The translocation strictly requires hydrolysis of ATP, because non-hydrolyzable ATP analogs do not promote peptide transport. ATP and ADP have similar affinities for TAP [30,31]; therefore, peptide translocation can be inhibited by ADP. The ATPase activity of TAP is substrate-specific and tightly coupled to peptide binding, indicating that peptide binding is a prerequisite for ATP hydrolysis, thereby possibly preventing waste of ATP without transport of peptides (S. Gorbulev and R. Tampé, manuscript in preparation). A structural rearrangement of the NBDs seems to function as a molecular switch to activate the ATPase of TAP. At the moment, there are no data available, if the NBDs are equal in function and if they work in a sequential or synchronic manner, thereby implying whether one or two ATPs are needed for substrate translocation.

6. Substrate specificity of TAP

The peptide specificity was analyzed by experiments based on trapping transported peptides in the ER by glycosylation and on ATP-independent binding assays (for review see [32]). Those peptides are translocated most efficiently into the ER, which are similar or slightly larger in length as expected for MHC class I binding. The contribution of each peptide residue to the affinity for TAP was determined by screening combinatorial peptide libraries [33]. With this method, the average affinity of a randomized peptide mixture with one common residue is compared to a totally randomized peptide mixture. The strongest differences were observed for the carboxy-terminal amino acid and the first three amino-terminal residues. The amino acids in between do not significantly contribute to substrate specificity. This binding motif combines maximal diversity in the epitope where T-cell receptor recognition occurs with maximal binding affinity for antigen processing. The matched preferences of human TAP and class I suggest a coevolution of the genes [32].

Although, TAP genes of all species are polymorphic, a functional polymorphism was so far only found for rat TAP [34] and for a human TAP2iso splice variant [35]. Mouse TAP, rat TAP from the $RT1^u$ strain and human TAP1/2iso were described to be selective for hydrophobic carboxy-termini of the peptide, whereas rat TAP from the $RT1^a$ strain and human TAP seem to be permissive for peptides containing hydrophobic and basic carboxy-termini [36,37].

7. Loading of peptides from TAP onto MHC molecules

The assembly and loading of MHC class I molecules require a number of other proteins (for review see [3]). At least four proteins – calnexin, calreticulin, ERp57 and tapasin – are involved in the assembly of heavy chain and β_2-microglobulin. The MHC class I molecules interact with the TAP complex via an ER-resident type I glycoprotein, named tapasin (Fig. 1A) [38,39]. Tapasin mediates complex formation and the crosstalk of structural information of MHC and TAP and is therefore important for class I assembly and editing. Tapasin has two independent functions: First, it increases the level of TAP, thereby increasing the efficiency of peptide transport and, secondly, it associates with MHC class I molecules, thereby facilitating directly loading and assembly of class I molecules [40,41]. The MHC-peptide complexes, which are kinetically stable, can leave the ER to the cell surface.

8. TAP and human diseases

Some viruses are known to interfere with antigen presentation in infected cells (for review see [42]). For example, the immediate early gene product ICP47 of herpes simplex virus type 1 inhibits peptide transport into the ER by blocking peptide binding to TAP [43–46]. The late expressed ER-resident transmembrane class I glycoprotein US6 from human cytomegalovirus inhibits peptide translocation probably by binding to the ER-luminal part of TAP, because neither peptide binding nor ATP binding to TAP seems to be affected [47–49].

There is little knowledge about human TAP defects. The homozygous TAP2 −/− siblings of one family have reduced expression of MHC class I molecules on the cell surface and reduced amounts of cytotoxic T-lymphocytes. On the other hand, these people do not show an increased susceptibility to viral infections. The antigens seem to be transported into the ER lumen in a TAP-independent manner, although under normal conditions TAP appears to be the dominant pathway [50]. In some tumor tissues, a down-regulation of TAP mRNA by an unknown mechanism or mutation of TAP was observed [51,52]. The suppression of TAP may be a mechanism for tumor cells to escape the immune response.

The TAPs as peptide transporters are a key link between antigen processing and presentation. However, many questions remain open to fully understand the whole process of T-cell immunity. Further analysis will clarify the significance of TAP defects or down-regulation in tumors and, in particular, the role in the interaction of malignant cells with the immune system of the host.

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Immunology

Immunophenotyping of melanomas for tyrosinase: Implications for vaccine development

(tumor immunology/cancer vaccine)

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Contributed by Lloyd J. Old, May 24, 1995

ABSTRACT **Tyrosinase (EC 1.14.18.1), the key enzyme in melanin synthesis, has been shown to be one of the targets for cytotoxic T-cell recognition in melanoma patients. To develop serological reagents useful for immunophenotyping melanoma for tyrosinase, human tyrosinase cDNA was expressed in an *Escherichia coli* expression vector. The purified recombinant tyrosinase was used to generate mouse monoclonal and rabbit polyclonal antibodies. The prototype monoclonal antibody, T311, recognized a cluster of protein moieties ranging from 70 to 80 kDa in tyrosinase mRNA-positive melanoma cell lines and melanoma specimens as well as in L cells transfected with tyrosinase cDNA. Untransfected L cells and L cells transfected with tyrosinase-related protein 1, TRP-1(gp75), were nonreactive. Immunohistochemical analysis of melanomas with T311 showed tyrosinase in melanotic and amelanotic variants, and tyrosinase expression correlated with the presence of tyrosinase mRNA. Melanocytes in skin stained with T311, whereas other normal tissues tested were negative. The expression pattern of three melanosome-associated proteins—tyrosinase, TRP-1(gp75), and gp100—in melanoma was also compared. Tyrosinase and gp100 are expressed in a higher percentage of melanomas than TRP-1(gp75), and the expression of these three antigens was discordant. Tyrosinase expression within individual tumor specimen is usually homogenous, distinctly different from the commonly observed heterogenous pattern of gp100 expression.**

Cutaneous melanin pigments are synthesized by melanocytes. The biosynthesis of melanin pigments involves a family of enzymes, including tyrosinase, tyrosinase-related-protein 1 (TRP-1), and tyrosinase-related-protein 2 (TRP-2) (1). Of these, tyrosinase (EC 1.14.18.1) is the principal enzyme, and mutations of the tyrosinase gene have been documented in various forms of albinism (2, 3).

The tyrosinase gene is composed of five exons, with the predicted amino acid sequence containing a leader signal peptide and a transmembrane domain (4, 5), consistent with it being a protein anchored to the membrane of the melanosomes. As a melanocyte differentiation antigen, tyrosinase is commonly expressed in malignant melanoma, as initially demonstrated by enzymatic 3,4-dihydroxyphenylalanine (dopa)-oxidase reaction (6) and subsequently by tyrosinase mRNA studies (7). Two recent reports indicated that tyrosinase is recognized by cytotoxic T cells from melanoma patients, one in the context of HLA-A2 (7) and the other in the context of HLA-A24 (8). The tyrosinase sequences recognized by HLA-A2-restricted cytotoxic T cells have been mapped to two nanopeptides, located in the signal peptide and in the catalytic domain (9). These findings open the way to immunotherapeutic strategies using tyrosinase peptides and/or protein. In this paper, we describe the production of recombinant tyrosinase protein and polyclonal and monoclonal antibodies (mAbs) that recognize tyrosinase. The frequency and characteristics of tyrosinase expression in melanomas were compared to the expression of two other melanosome-associated proteins, gp100 (10) and TRP-1(gp75) (11).

MATERIALS AND METHODS

Cell Lines and Tissues. Melanoma cell lines have been described previously (12–14), and cultured melanocytes were kindly provided by M. Eisinger (Lederle Laboratories, Pearl River, NY). L cells transfected with human tyrosinase or TRP-1(gp75) cDNA (15) were kindly provided by A. Houghton (Memorial Sloan–Kettering Cancer Center). Specimens of normal and tumor tissues were obtained from the Departments of Pathology at the New York Hospital–Cornell Medical Center and Memorial Sloan–Kettering Cancer Center.

mAbs. TA99, a mouse mAb against human gp75, has been described (11). HMB45, a mAb reactive with melanoma antigen gp100 (10), was obtained commercially (Dako).

Oligonucleotide Synthesis. Oligonucleotides were synthesized based on the published sequences (4) to amplify the tyrosinase gene. The 5′ primer, derived from exon 1, corresponds to the N-terminal sequence after cleavage of the signal peptide (5′-CACACGGATCCGATGACGATGACAAAGC-CTGTGTCTCCTCTAAGAACC-3′, designated as tyrA). In addition to a *Bam*HI restriction site, this primer also contains the DNA sequences encoding an enterokinase cleavage site (Asp-Asp-Asp-Asp-Lys). Two 3′ primers were prepared, one derived from exon 5 (5′-CACACAAGCTTGATC-CGACTCGCTTGTTCC-3′, designated as tyrB5), ending 5′ to the transmembrane domain sequences. The other 3′ primer was derived from exon 4 (5′-CACACAAGCTTGCCTTTG-GAGCCACTGCTC-3′, tyrB4). Both 3′ primers include a *Hin*dIII restriction site for cloning. All oligonucleotides were synthesized commercially (Operon Technologies, Alameda, CA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was prepared from melanoma cell lines or melanoma specimens, and tyrosinase mRNA expression was evaluated by RT-PCR using 35 PCR cycles and an annealing temperature of 60°C.

Prokaryotic Expression Cloning in *Escherichia coli*. PCR-amplified tyrosinase cDNA was cloned into expression vector pQE9 (Qiagen, Chatsworth, CA). Recombinant protein was induced by isopropyl β-D-thiogalactoside and purified by Ni^{2+} affinity chromatography, following the manufacturer's protocol (Qiagen). Protein was monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis and silver staining.

Generation of Mouse Hybridomas and Rabbit Antisera. Purified recombinant tyrosinase was used to immunize

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Abbreviations: mAb, monoclonal antibody; RT-PCR, reverse transcription polymerase chain reaction; dopa, 3,4-dihydroxyphenylalanine.

BALB/c mice, and the spleen cells were fused with mouse myeloma cell line SP2/0. Hybridomas were generated and cloned as described (13), and screened by solid-phase ELISA using the immunizing fusion protein as the target antigen. A panel of melanoma antigens—e.g., MAGE-1 (16), expressed in the same vector—was used as a negative control for screening.

Antisera from rabbits immunized with the same recombinant protein were prepared by HRP (Denver, PA).

Immunoblotting. Immunoblotting was performed using a chemiluminescent detection system (Amersham) as described (16).

Immunohistochemical Procedures. The avidin-biotin complex immunoperoxidase procedure was carried out as described (17), using biotinylated secondary antibodies and avidin-biotin horseradish peroxidase complex. Diaminobenzidine tetrahydrochloride was used as the chromogen. In the case of pigmented tissues such as normal skin and melanomas, the secondary antibody step was followed by incubation with streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim) and visualized with new fuchsin substrate. This second method generates a red reaction product, easily distinguishable from the brown-black melanin pigment. Isotype-matched unrelated immunoglobulins (Becton Dickinson) were used as negative controls. Immunofluorescent assays on transfected L cells were performed as described (18).

RESULTS

Typing of Melanoma Cell Lines and Melanoma Specimens for Tyrosinase mRNA. Twelve melanoma cell lines and 38 melanoma samples were evaluated for tyrosinase mRNA expression by RT-PCR amplification, using two sets of primer combinations—i.e., tyrA/tyrB4 and tyrA/tyrB5. Both primer pairs gave the same result. Of 12 melanoma cell lines, 7 (58%) were positive, and 5 were negative. Of 38 melanoma samples, 32 (84%) were positive, including grossly amelanotic melanomas. Four melanomas were negative, and 2 showed equivocal signals.

Production of Recombinant Tyrosinase Protein. Total RNA was prepared from cultured normal melanocytes. RT-PCR with tyrA/tyrB5 revealed the expected 1393-bp product, encoding 452 of the 511 amino acids in the mature tyrosinase molecule. This cDNA product was digested with *Bam*HI and *Hin*dIII and cloned into pQE9. The inserted tyrosinase sequence was confirmed by DNA sequencing. Isopropyl β-D-thiogalactoside induction of the transformed clones resulted in the synthesis of recombinant protein with an apparent molecular mass of ≈52 kDa, consistent with the predicted molecular mass. This recombinant tyrosinase contained hexahistidine at the N terminus and was purified by Ni^{2+} affinity column chromatography (Fig. 1).

Mouse mAbs. Mouse mAbs were generated against affinity-purified tyrA/tyrB5 recombinant protein and screened by ELISA. Clones secreting mAb showing reactivity toward recombinant tyrosinase but not against human MAGE-1 expressed in the same vector were harvested and subcloned. After three subclonings, seven clones were isolated—T41, T72, T125, T311, T550, T562, and T620. Each of the seven clones produced mAb with ELISA titer of 1:8000 to 1:32,000 against recombinant tyrosinase but no cross-reactivity with recombinant MAGE-1.

Rabbit Polyclonal Antisera Against Tyrosinase. The tyrA/tyrB5 recombinant protein was also used to immunize rabbits for polyclonal antisera. The polyclonal antisera showed the same reactivity pattern as the mouse mAbs by ELISA and immunoblotting analysis (Fig. 2*c*, see below).

Immunoblotting with Melanoma Cell Line Lysates. Nonidet P-40 lysates were prepared from SK-MEL-19, SK-MEL-30, and MZ2-MEL3.1. RT-PCR showed that SK-MEL-19 and SK-MEL-30 express tyrosinase mRNA, whereas MZ2-MEL3.1 does not. Immunoblotting with the seven mouse mAbs generated against tyrosinase showed a similar reactivity pattern, with the major antigenic species consisting of a cluster of proteins ranging from 70 to 80 kDa (Fig. 2*a*). A minor species of 55 kDa can be seen in cell lines that are strongly reactive—e.g., SK-MEL-19. T311, an IgG2a mAb, was selected as the prototype reagent because of its strong reactivity and was used in all subsequent experiments.

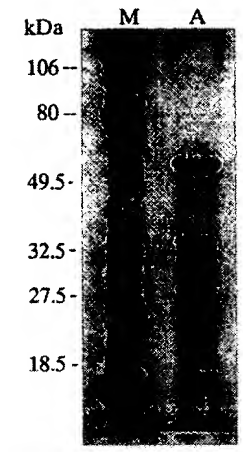

FIG. 1. Silver-stained gel of affinity-purified tyrA/tyrB5 tyrosinase recombinant protein (lane A), showing the main species at ≈52 kDa. Lane M, molecular mass standards.

Four additional cell lines were then tested with T311, including two tyrosinase mRNA-positive lines, SK-MEL-13 and SK-MEL-37, and two mRNA-negative lines, SK-MEL-187 and MZ2-MEL2.2. Results showed positive immunoblotting in the two mRNA-positive lines but not in mRNA-negative lines.

L Cells Expressing Tyrosinase React with T311. To prove that the protein species detected in immunoblotting were tyrosinase products, L cells transfected with the tyrosinase gene (15) were tested. Results showed that L-cell transfectants

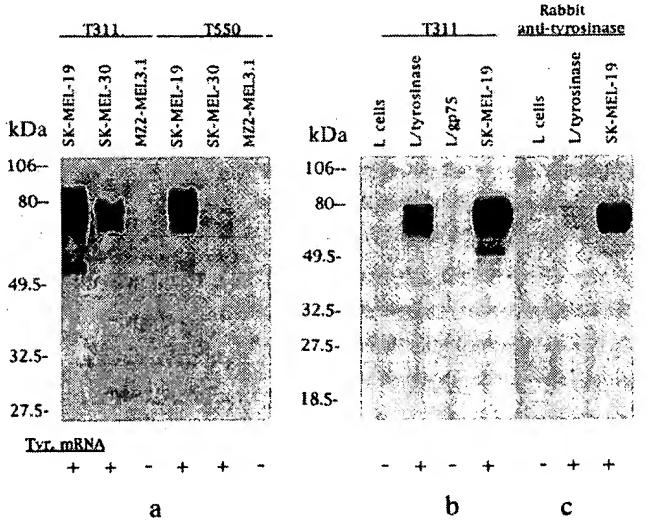

FIG. 2. Immunoblot analysis of anti-tyrosinase antibodies against cell line lysates correlated with tyrosinase mRNA expression by RT-PCR (bottom). (*a*) Two representative mAbs, T311 and T550, tested on three melanoma cell lines. The major reacting bands are in the range of 70–80 kDa, and a minor 55-kDa band is seen in SK-MEL-19. (*b*) L cells transfected with tyrosinase gene show a reactive pattern similar to tyrosinase-positive melanoma cell lines (e.g., SK-MEL-30 in *a*), whereas untransfected L cells and gp75-transfected L cells are negative. (*c*) Rabbit anti-tyrosinase polyclonal serum shows a similar reactivity pattern.

displayed an immunoblotting pattern similar to that of tyrosinase-positive melanoma cell lines (Fig. 2*b*)—i.e., a cluster of protein species at the range of 70–80 kDa. The 55-kDa species was not observed in the transfected L cells. Immunofluorescent assays with T311 showed positive reactivity in transfected L cells and no reactivity in the untransfected L cells.

T311 Does Not React with TRP-1(gp75). To rule out the possibility that T311 cross-reacts with TRP-1(gp75), L cells transfected with the TRP-1 (15) gene were tested. Immunofluorescent staining with TA99, a previously described anti-gp75 mAb, showed strong staining of these cells, confirming the expression of gp75 by L-cell transfectants. In contrast, immunofluorescent and immunoblotting assays of these transfectants with T311 were negative (Fig. 2*b*), indicating no cross-reactivity of T311 with TRP-1(gp75).

Immunohistochemical Reactivity of T311. T311 was tested against cytological preparations of SK-MEL-19, SK-MEL-187, and MZ2-MEL3.1 by the avidin-biotin complex immunoperoxidase procedure. Results showed positive cytoplasmic staining in the tyrosinase mRNA-positive line, SK-MEL-19, but not in the tyrosinase mRNA-negative lines. Strongest staining was observed in the perinuclear region, with the dendritic processes showing weaker reactivity. Frozen sections of normal skin from Caucasian and African-American individuals were then tested. Melanocytes from both specimens showed intense cytoplasmic staining, whereas pigmented basal keratinocytes and other cell types were negative (Fig. 3*a*). All noncutaneous normal human tissues tested were negative, including cerebral cortex, cerebellum, lung, kidney, spleen, colon, stomach, uterus, testis, skeletal muscle, smooth muscle, and adipose tissue. Normal retinal tissue, known to express tyrosinase (7), was unavailable for evaluation.

A panel of 16 melanoma specimens was tested, 13 tyrosinase mRNA-positive and 3 tyrosinase mRNA-negative, as defined by RT-PCR. Of the 13 tyrosinase mRNA-positive cases, 11 were amelanotic based on evaluation of the hematoxylin/eosin-stained sections. Eleven of 13 tyrosinase mRNA-positive cases showed positive cytoplasmic staining (Fig. 3*b*), whereas the three tyrosinase mRNA-negative cases were negative for T311 staining (Table 1). The two mRNA-positive, T311-nonreactive cases were found to have low or equivocal levels of tyrosinase mRNA.

Expression of Tyrosinase, TRP-1(gp75), and gp100 in Melanomas. With the same panel of 16 melanomas (see above), tyrosinase expression was compared to expression of two other melanocyte antigens, namely TRP-1(gp75) (detected by TA99) and gp100 (detected by mAb HMB45) (Table 1). TRP-1(gp75) was expressed in 8, tyrosinase in 11, and gp100 in 12 of the 16 cases. Detailed comparison of the cases indicated that the expression of these three antigens was discordant. The disso-

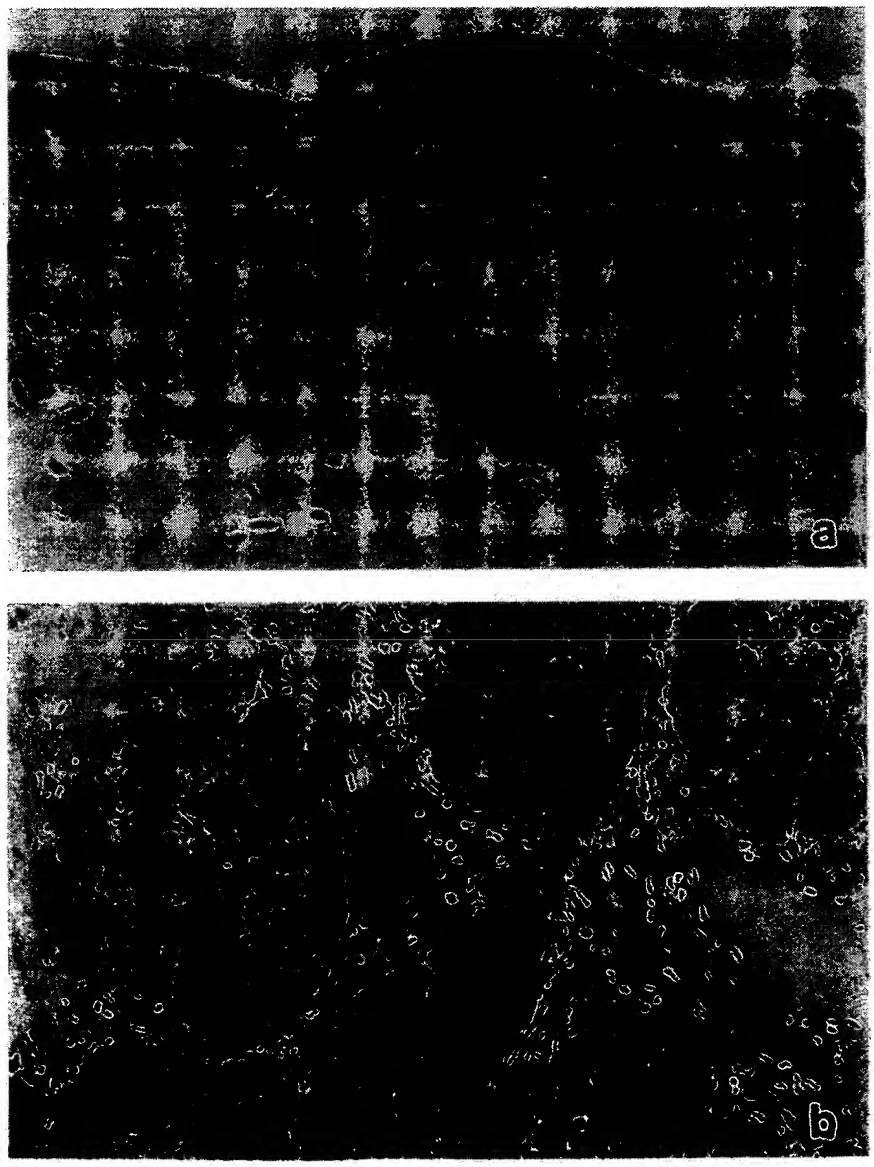

FIG. 3. Immunohistochemical reactivity of T311 with normal skin (*a*) and a melanoma specimen (*b*). Normal skin shows intense staining of the melanocytes (×360), and the melanoma specimen shows positive homogenous staining of the tumor cells (×180) (red, with new fuchsin substrate).

Table 1. Expression of tyrosinase, TRP-1, and gp100 in 16 melanoma specimens

Specimen	Tyrosinase mRNA*	Tyrosinase T311†	TRP-1 TA99†	gp100 HMB45†
1	+	++	+++	+/++‡
2	−	−	−	−
3	+	−	−	−
4	+	++	+++	+++
5	+++	++	−/++	(−/+)
6	+	+	(−/++)	+++
7	+++	+/++	−/+	+++
8	+++	+++	++/+++	++
9	+++	+++	−	(−/++)
10	+++	++	−	(−/+)
11	−	−	−	−
12	−	−	−	−
13	+++	+	−	+++
14	+	−	+++	(−/++)
15	+++	+++	−	+++
16	+++	+++	++/+++	+++

Parentheses indicate cases with <20% of tumor cells stained.
*mRNA expression was determined by the quantity of RT-PCR products, judged by the intensities in ethidium bromide-stained gels.
†Antigenic expression was scored as −, +, ++, and +++ subjectively, based on the staining intensity.
‡Intratumor staining heterogeneity is indicated by two different scores in one entry.

ciation between TRP-1(gp75) and tyrosinase expression (four cases $TA99^{-}T311^{+}$ and one case $TA99^{+}T311^{-}$) adds further proof that T311 does not react with TRP-1(gp75).

Another significant difference concerns the heterogeneity of antigenic expression within individual tumor specimen. In contrast to tyrosinase-positive cases, where the staining intensity appears to be uniform among tumor cells, staining with HMB45 and TA99 was more heterogenous. This intratumor heterogeneity is particularly apparent in gp100 expression, as one-third (4 of 12) of the $HMB45^{+}$ cases showed positive staining in <20% of the tumor cells.

DISCUSSION

Two types of melanin pigments are produced by melanocytes—i.e., the black-brown eumelanin and the red-yellow pheomelanin (1). Tyrosinase is the key enzyme in the synthesis of both pigments, catalyzing two initial steps in the biosynthetic pathway—i.e., hydroxylation of tyrosine to dopa and oxidation from dopa to dopaquinone. Dopaquinone then enters two separate pathways, leading to the synthesis of eumelanin or pheomelanin. In addition to tyrosinase, several other structurally related proteins are encoded by the tyrosinase gene family, notably tyrosinase-related protein 1 (TRP-1) (19, 20) and tyrosinase-related protein 2 (TRP-2) (21–23). These two molecules are also involved in the melanin synthesis pathway, both of them contributing more to the synthesis of eumelanin than to pheomelanin (24–26). TRP-1 and TRP-2 share moderate sequence similarities with tyrosinase, the amino acid homology with the tyrosinase being 43% and 40%, respectively (22, 25).

The molecular mass of tyrosinase has been reported to range from 60 kDa (27) to 75 kDa (25, 28), as determined by enzyme-based assays in the earlier literature (29) and subsequently by immunoprecipitation or immunoblotting methods using antibodies (27, 28, 30). The predicted molecular mass of the primary translation product of the tyrosinase gene is ≈58 kDa (4, 31), which, after processing and glycosylation, leads to the mature tyrosinase molecules in the melanosomes, with a microheterogenous mass of 70–75 kDa. Variation in the size of the mature tyrosinase product has been attributed to the presence of isozymic forms (29) and/or alternate splicing of tyrosinase mRNA (31–33). With the anti-tyrosinase mAb T311 generated in the present study, we detected a cluster of proteins at the 70- to 80-kDa range, consisting of at least three or four species. These species are clearly encoded by the tyrosinase gene because transfection of L-cell fibroblasts with the human tyrosinase gene led to their expression. The detection of these multiple tyrosinase species by T311 is likely due to the fact that our series of tyrosinase mAbs was generated against the unglycosylated peptide backbone synthesized in *E. coli*, and the recognized antigenic epitope would therefore be present in mature tyrosinase as well as in precursor and intermediate forms. In accord with this idea, a minor 55-kDa product was present in cell lines expressing higher levels of tyrosinase, possibly representing the primary unglycosylated translation product.

Antibodies against mammalian tyrosinase have previously been produced, including polyclonal antibodies against hamster tyrosinase (34), mouse tyrosinase (30), and human tyrosinase (15). Two mAbs have also been described, against mouse T_4 tyrosinase (30) and human tyrosinase (27). mAb 5C12, described by McEwan *et al.* (27), was generated by immunization with a fraction of a human melanoma cell line lysate enriched for tyrosinase activity and was reported to recognize an antigenic epitope residing in the carbohydrate moiety of tyrosinase. A protein species of ≈60 kDa with tyrosinase activity was immunoprecipitated by 5C12, but an additional 70-kDa species with tyrosinase activity was not recognized by 5C12. The reactivity of 5C12 for tyrosinase-related proteins is not known, and no immunohistochemical analysis of its reactivity with melanoma has been reported.

The conclusion that T311, the mAb generated in this study, is specific for the tyrosinase gene product comes from several lines of evidence. (*i*) T311 reactivity with melanoma cell lines and melanoma specimens cotypes with tyrosinase mRNA expression. (*ii*) T311 shows strong positive staining of melanocytes in immunohistochemistry but does not react with pigmented keratinocytes or other normal tissues tested. (*iii*) T311 immunoreacts with the same spectrum of proteins from L cells transfected with the tyrosinase gene as it does from tyrosinase-expressing melanoma cells. Cross-reactivity of T311 with TRP-1 products was ruled out for the following reasons. (*i*) T311 reactivity in melanoma specimens does not cotype with TRP-1 expression (as determined by mAb TA99). (*ii*) T311 does not react with L cells transfected with the TRP-1 gene. Because of the lack of suitable reagents, we are unable at present to exclude reactivity of T311 with TRP-2. However, the cotyping results between tyrosinase mRNA expression and mAb reactivity, and modest homology between TRP-2 and tyrosinase (40%), argue against the likelihood of such cross-reactivity.

Because of the recent demonstration that tyrosinase gene products can be recognized by cytotoxic T cells (7, 8) and helper T cells (35) in humans, there is considerable interest in tyrosinase as an antigenic target for melanoma vaccines. Due to variation in the level of tyrosinase expression in melanomas from different patients, it would be important to define a standard typing method for tyrosinase. Three approaches can be considered—one by histologic evaluation of pigment production in tumor cells, the second by analyzing tyrosinase mRNA expression by RT-PCR, and the third by immunohistochemical staining with anti-tyrosinase antibody. Of these methods, evaluation of pigment production is the least reliable, since melanomas producing only pheomelanin will often be considered amelanotic, and it is indeed well known to pathologists that melanin pigments have been commonly found in amelanotic lesions by staining with ammoniated silver nitrate, such as the Fontana–Masson stain (6). Our present study further proves this point by demonstrating that most amelanotic tumors are positive for tyrosinase mRNA and protein.

The second method, typing tyrosinase expression at the RNA level, although sensitive, may be suboptimal for clinical specimens for the following reasons: (*i*) mRNA expression may not consistently correlate with protein expression, (*ii*) the possible heterogeneity of tyrosinase expression within individual tumors cannot be evaluated by RT-PCR assay, and (*iii*) RT-PCR may not reliably assess the levels of tyrosinase mRNA expression in melanoma, due to the limited reliability of RT-PCR in quantitative assays as well as the dilutional effect of RNA from adjacent nonneoplastic tissues. In comparison, immunohistochemical phenotyping with T311 provides a simple reliable alternative for evaluating tyrosinase expression in a semiquantitative fashion. Analysis of a series of melanomas revealed T311 staining in all tyrosinase mRNA-positive cases, with the exception of two cases that showed low tyrosinase mRNA expression and no T311 reactivity, presumably reflecting low antigen expression. Although this result indicated that T311 staining was not as sensitive as RT-PCR, it could also be argued that tumors expressing antigen in such low density may not be an effective target for tumor vaccination.

With regard to selecting patients for vaccine trials, it is clear that immunophenotyping melanomas for antigens such as tyrosinase gives no direct information about presence or level of T-cell-recognized peptides presented on the cell surface. However, it seems reasonable to assume that high homogenous expression of antigen is a desirable characteristic and that antigen expression would likely correlate with levels of presenting peptides. The value of this immunophenotyping approach becomes evident as we compare results with other antigens that are potential vaccine targets. In addition to tyrosinase, six other melanoma antigens have been shown to be recognized by host cytotoxic T cells—i.e., MAGE-1 (36), MAGE-3 (37), Melan-A/MART-1 (38, 39), gp100 (40), TRP-1(gp75) (41), and BAGE-1 (42), and mAbs are now available for three of them—namely, MAGE-1 (MA454, ref. 16 and 77B, ref. 43), gp100 (HMB45), and TRP-1(gp75) (TA99). The MAGE-1 mAbs have not been shown to be useful for immunohistochemical typing of melanomas. Comparison of the protein expression patterns of tyrosinase, TRP-1(gp75), and gp100 in melanomas reveals two important differences. First, tyrosinase and gp100 were expressed by a high percentage of melanomas, including amelanotic variants. In comparison, TRP-1(gp75) expression was less frequent. This observation parallels the expression profile of their mRNA species (41). Second, tyrosinase expression appears to be homogenous within individual tumors, which is significantly different from the heterogenous expression of gp100, seen by us and others (10). The expression pattern of gp75 appears to be intermediate with regard to heterogeneity. These two features of tyrosinase expression—expression by many melanomas and a homogenous pattern of expression—suggest that tyrosinase may be a favorable antigenic target for tumor vaccination. In addition, given the heterogeneic expression of some melanocyte differentiation antigens in melanomas, as we demonstrate here, immunophenotyping of individual tumors will likely be a critical step in evaluating patients for tumor vaccination.

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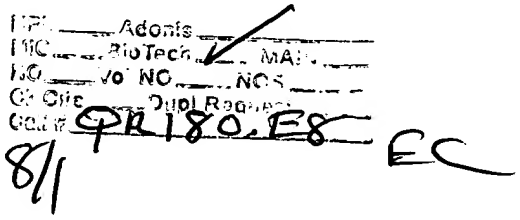

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A tyrosinase nonapeptide presented by HLA-B44 is recognized on a human melanoma by autologous cytolytic T lymphocytes

The human tyrosinase gene has been reported previously to code for two distinct antigens recognized on HLA-A2 melanoma cells by autologous cytolytic T lymphocytes (CTL). By stimulating lymphocytes of melanoma patient MZ2 with a subclone of the tumor cell line of this patient, we obtained a CTL clone that lysed this subclone but did not lyse other subclones of the same melanoma cell line. The sensitive melanoma subclone was found to express a much higher level of tyrosinase than the others, suggesting that the antigen recognized by the CTL might be encoded by tyrosinase. Transfection of a tyrosinase cDNA demonstrated that the CTL clone indeed recognized a tyrosinase product presented by HLA-B*4403. The relevant antigenic peptide corresponds to residues 192–200 of the tyrosinase protein. Lymphoblastoid cells of the B*4402 subtype were not recognized by the CTL following incubation with the peptide. Nevertheless, by stimulating *in vitro* lymphocytes of a healthy HLA-B*4402 donor with autologous adherent cells pulsed with the same peptide, we obtained a CTL clone which recognized tumor cells expressing tyrosinase and HLA-B*4402. As HLA-B44 is expressed in 24% of Caucasians, the tyrosinase-B44 antigen may constitute a useful target for specific immunotherapy of melanoma.

1 Introduction

Cultures of irradiated tumor cells mixed with blood lymphocytes of the same patient can produce responder lymphocyte populations that display cytolytic activity against the tumor cells [1–3]. By limiting dilution of these responder cell populations, it is possible to obtain cytolytic T lymphocyte (CTL) clones that lyse the tumor cells but do not lyse autologous EBV-transformed lymphoblastoid cells, autologous fibroblasts or natural killer targets such as K562 [2, 4, 5]. A number of genes coding for tumor antigens recognized by autologous CTL clones on melanoma cells have been identified. A first group of genes, which belong to three gene families named MAGE, BAGE and GAGE, is expressed in a significant proportion of tumors of various histological types [6–12]. These genes are not expressed in normal tissues except testis. A second group of genes codes for differentiation proteins that are specific for the melanocytic lineage. They are expressed both in normal melanocytes and in melanoma cells. This group of genes comprises tyrosinase, which codes for two antigens recognized by CTL on almost all HLA-A2 melanomas [13, 14], Melan-$A^{MART\text{-}1}$, $gp100^{Pmel17}$ and $gp75^{TRP\text{-}1}$ [15–19].

An extensive study of CTL clones of patient MZ2 that are directed against autologous melanoma cell line MZ2-MEL has been performed [5]. The lytic activity of a number of these CTL clones was examined on several clonal sublines derived from the initial tumor cell line, which was not clonal. The results led to a subdivision of the panel of CTL into three groups [20].

A first group recognized antigens expressed by all the sublines tested. Selections with these CTL clones *in vitro* produced MZ2-MEL cell variants that were resistant to some of these CTL, but not to others, and this led to the conclusion that the MZ2-MEL sublines expressed at least four distinct antigens. Two of these antigens are presented by HLA-A1 and are encoded by genes MAGE-1 and MAGE-3, respectively [21, 22]. Another antigen that is recognized on HLA-Cw6 is encoded by two members of the GAGE gene family [8].

A second group of CTL recognized an antigen which was present on the initial tumor cell line but not on a subline that had undergone more than 150 culture transfers. This subline had lost the expression of HLA-A29, B44 and Cw16. Two of the CTL clones that failed to recognize were found to be directed against antigens presented by HLA-Cw16. One antigen is encoded by MAGE-1 [23]. The other is encoded by a gene of the BAGE family [7].

A third group of CTL was obtained after stimulation with subclone MZ2-MEL.43, which had been isolated after mutagen treatment of clonal line MZ2-MEL3.0. These CTL recognized an antigen present on subclone MZ2-MEL.43, but neither on MZ2-MEL3.0 nor on other sublines. We then derived a large number of subclones from the original tumor cell line MZ2-MEL. The antigen was detected on approximately 5% of these subclones. We report here that this antigen, named MZ2-C, is encoded by the tyrosinase gene.

[I 14998]

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2 Materials and methods

2.1 Cell lines

The melanoma cell line MZ2-MEL was derived from an abdominal metastasis of patient MZ2. Subclone MZ2-MEL.3.0 was obtained by limiting dilution. Subline MZ2-

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MEL.43 was derived by limiting dilution from MZ2-MEL.3.0 cells that had survived to a mutagen treatment [5, 20]. The origins of the MZ2-MEL sublines and of cell lines LB33-MEL.A and SK29-MEL were previously described [20, 23–25]. Melanoma cell line LG2-MEL was a gift from Dr. G. Degiovanni (Université de Liège, Liège, Belgium). All melanoma cell lines were grown in Iscove's medium supplemented with 10% FCS. COS cells were grown in DME medium containing 10% FCS. EBV-transformed cell lines were obtained by transformation of peripheral blood lymphocytes with EBV and cultured in Iscove's medium supplemented with 10% FCS. BI2-EBV is a gift from Dr. Rickinson (Birmingham, GB).

Autologous CTL clone 22/31 was derived from peripheral blood lymphocytes of patient MZ2 and grown in conditions similar to those previously described [20]. CTL IVSB was derived from patient SK29(AV) and grown as described [25]. Autologous CTL clone 329B/5 was derived by stimulation *in vitro* as follows. Adherent cells from PBL of healthy donor LB-1161 (HLA-B*4402 positive) were grown for 1 week in RPMI medium supplemented with 10% FCS, IL-4 (50 U/ml) and GM-CSF (100 ng/ml). These cells were pulsed on 24-well plates (Nunc, Roskilde, Denmark) with peptide SEIWRDIDF (50 μM) for 4 h in the presence of β2-microglobulin (2.5 μg/ml, Sigma). After irradiation of the adherent cells, 2×10^6 $CD8^+$ sorted T lymphocytes were added in a final volume of 2 ml Iscove medium supplemented with 10% human serum, 1000 U/ml IL-6 and 5 ng/ml IL-12. The responder cells were stimulated on days 7 and 14 with LB1161 adherent cells pulsed with the peptide as above in medium supplemented with 10 U/ml IL-2 and 5 ng/ml IL-7 (British Biotechnology). On day 21, responder lymphocytes were cloned by limiting dilutions in microwells containing 10^4 irradiated LB33-MEL.A pulsed with peptide SEIWRDIDF (1 μM) and 2×10^4 irradiated lymphoblastoid cell line LG2-EBV cells as feeder cells. The microcultures were stimulated every 7 days by the same procedure. After 5 weeks, the CTL clone 329B/5 was grown on 24-well plates and stimulated weekly with 2×10^5 irradiated LB33-MEL.A cells pulsed with the peptide and 10^6 irradiated LG2-EBV cells in culture supplemented with IL-2 (50 U/ml) and IL-4 (5 U/ml).

2.2 CTL stimulation assay

Tumor cell lines and COS-7 transfectants were tested for their ability to stimulate the production of TNF by CTL, as described [26]. Briefly, 2000 CTL were added in 100 μl Iscove's medium supplemented with 10% human serum and 25 U/ml IL-2 in microwells containing 15000–20000 target cells. After 24 h, the supernatant was collected and its TNF content determined by testing its cytotoxicity for WEHI-164 clone-13 cells [27] in a colorimetric assay as described [28].

2.3 Transfection of COS-7 cells

Transfection experiments were performed by the DEAE-dextran-chloroquine method as described [13, 29]. Plasmid pcDNAI/Amp (100 ng; Invitrogen Corporation, San Diego, CA) containing the tyrosinase or a subgenic fragment was cotransfected with 100 ng of expression vector containing HLA-coding sequences. The HLA-A2.1 gene was cloned from a cosmid library prepared from DNA of lymphocytes of patient SK29(AV) [25]. The HLA-A1 gene was derived from another patient and provided by Dr. Girdlestone [30]. cDNA encoding HLA-B44, -A29, -B37, and -Cw16 were cloned from a cDNA library constructed with poly(A)$^+$ RNA extracted from MZ2-MEL.43 cells. COS cells were incubated for 24 h at 37°C and a CTL stimulation assay was performed.

2.4 Cloning of subgenic fragments of the tyrosinase gene

Tyrosinase cDNA clone 123.B2 was obtained from a cDNA library of SK29-MEL [13]. Fragments were generated by PCR amplification and incorporated into the EcoRV site of pcDNAI/Amp. As primers, we used: oligonucleotides VB34 (5′-CCGAATTCGCCATGTCTGAAATCTGG-3′) and VB29 (5′-AATCTAGACGCCTAGCTACAGACAATCTGCCA-3′) to generate fragment 574–831; oligonucleotides VB58 (5′-CGGGATCCGCCGCCATGCCAGAGAAGGAC-3′) and VB60 (5′-GCTCTAGAGCCTTATGCTTCATGGGC-3′) to generate fragment 385–612.

2.5 HLA-B44 subtyping

The melanoma and EBV cell lines were characterized for the HLA-B44 subtypes as follows: genomic DNA (0.5 μg) of the sample was amplified with oligonucleotides BX3S1 (5′-GGGTCCAGGGTCTCACATCA-3′) and BX3R1 (5′-CCAGGTATCTGCGGAGCG-3′) for 10 cycles (denaturation 30 s at 94°C, annealing 30 s at 65°C and extension 1 min at 72°C) followed by 25 cycles (30 s at 94°C, 30 s at 60°C, 1 min at 72°C). The fragment corresponding to a part of exon 3 was digested with restriction enzymes (RsaI, PvuII and BsaAI) to discriminate between subtypes -B*4402, 03, 04 and 05.

2.6 Peptide synthesis and peptide recognition assays

Peptides were synthesized on solid phase using Fmoc for transient N-terminal protection and characterized by amino acid analysis. Standard ^{51}Cr-release assays were used to determine lysis of target cells [31].

Target cells ($1 \times 10^6 - 2 \times 10^6$) were labeled with 100 μCi of $Na^{51}CrO_4$ in 30 μl FCS. For assays with CTL 329B/5, the cells were labeled in the presence of anti-MHC class I mAb W6/32. After 1-h incubation, the cells were washed twice, then incubated in the presence of various concentrations of the peptide in a total volume of 100 μl in conical 96-well microtiter plates (Greiner, Nürtingen, Germany) for 30 min. CTL were added in 100 μl. Cr release was measured after incubation at 37°C for 4 h.

2.7 Quantitative measurements of tyrosinase expression

Quantitative RT-PCR was essentially performed as described [11]. Briefly, isolation of total RNA was performed as reported [32]. cDNA was obtained by reverse

transcription performed on 2 μg of total RNA with an oligo(dT) primer. cDNA obtained from the clone SK29-MEL.1 was included pure and diluted in each assay of quantitative PCR and used as standard. cDNA corresponding to 100 ng of total RNA was amplified for 22 cycles by PCR. Trace amounts of [α-^{32}P]dCTP (0.2 μCi) were added and accurate quantitation was obtained using the phosphor-imaging technology (Phosphor-Imager, Molecular Dynamics, Sunnyvale, CA). Primers used for the amplification are VB17 (5′-GGATAGCGGATGCCTCTCAAAG-3′) located in exon 3 and VB18 (5′-CCCAAGGAGCCATGACCAGAT-3′) located in exon 5. The cycle conditions were: 94 °C 1 min, 65 °C 2 min and 72 °C 3 min. Expression of the various samples was reported as percentages of that found in SK29-MEL.1 after correction for RNA integrity by taking into account the expression level of the β-actin gene [11].

3 Results

3.1 High expression of the tyrosinase gene in the MZ2-MEL subline bearing antigen MZ2-C

We have observed that the tyrosinase gene is expressed in all melanoma tumor samples, whereas about 30 % of the melanoma cell lines are negative, suggesting that loss of expression of the tyrosinase gene can occur *in vitro* [13]. Quantitative PCR measurements of the expression of the tyrosinase gene were made on several sublines derived from melanoma cell line MZ2-MEL. The level of expression of these sublines was low, ranging from 1 % to 8 % of that observed with the tyrosinase-positive cell line SK29-MEL, except for subline MZ2-MEL.43 which expressed 120 % (Table 1). This observation caught our attention because MZ2-MEL.43 was the only subline that expressed antigen MZ2-C recognized by autologous CTL clone 22/31 which lysed this subline but failed to lyse any other MZ2-MEL subline (Fig. 1).

Table 1. Expression of the tyrosinase gene in MZ2-MEL sublines[a)]

Clone	Expression of tyrosinase (percent relative to SK29-MEL)
MZ2-MEL.43	119
MZ2-MEL 3.0	8
MZ2-MEL 3.1	< 1
MZ2-MEL 2.2	2
MZ2-MEL 2.2.5	< 1
MZ2-MEL 61	1

a) cDNA synthesis and quantitative PCR amplification were performed as described in Sect. 2.7. The results were normalized for the expression of β-actin. They are expressed as the percentage of the level of expression of melanoma line SK29-MEL. The origin of the MZ2-MEL sublines is described in Sect. 2.1.

3.2 Antigen MZ2-C is encoded by the tyrosinase gene and presented by HLA-B44

To test the possibility that anti-MZ2-C CTL 22/31 recognized a tyrosinase-encoded peptide, we cotransfected into COS-7 cells a tyrosinase cDNA cloned in expression vector pcDNAI/Amp together with a construct containing the coding sequences of either HLA-A1, A29, B37, B44 or Cw16, all being HLA alleles of patient MZ2. One day after the transfection, COS cells were incubated with CTL 22/31. Antigen recognition by the CTL was assessed by measuring the amount of TNF released after 24 h. A very significant amount of TNF was produced by CTL 22/31 stimulated with COS cells transfected with the tyrosinase sequence and with HLA-B44 (Fig. 2). No stimulation was observed with COS cells transfected with HLA-B44 alone or with the combination of tyrosinase and one of the other HLA clones.

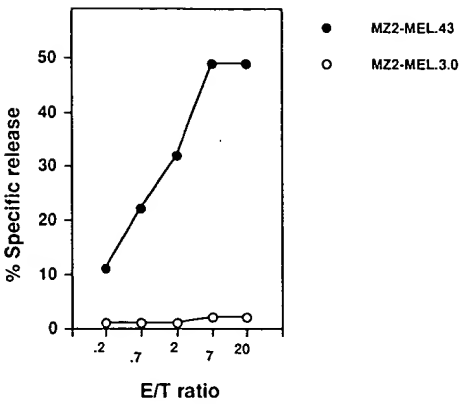

Figure 1. Lysis of MZ2-MEL.43 by CTL clone 22/31. The cytolytic activity was measured in a 4-h ^{51}Cr-release assay. MZ2-MEL.3.0 is a subclone of the original tumor cell line MZ2-MEL. MZ2-MEL.43 is a clonal subline derived from a mutagenized MZ2-MEL.3.0 culture.

3.3 Identification of the antigenic peptide

We resorted to a genetic approach to localize the tyrosinase sequence coding for antigen MZ2-C. Various fragments of the tyrosinase cDNA were obtained by digestion

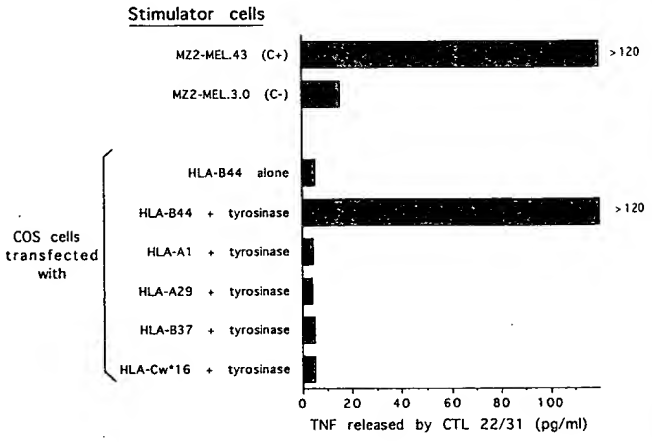

Figure 2. Expression of antigen MZ2-C by COS cells cotransfected with the tyrosinase and HLA-B44 coding sequences. COS-7 cells were cotransfected with expression vectors containing the tyrosinase sequence and the coding sequence of different class I alleles expressed by melanoma MZ2-MEL. One day later, 2000 cells of CTL 22/31 were added to 30 000 transfected cells. After 24 h, the amount of TNF in the supernatant was measured by its toxicity on WEHI-13 cells.

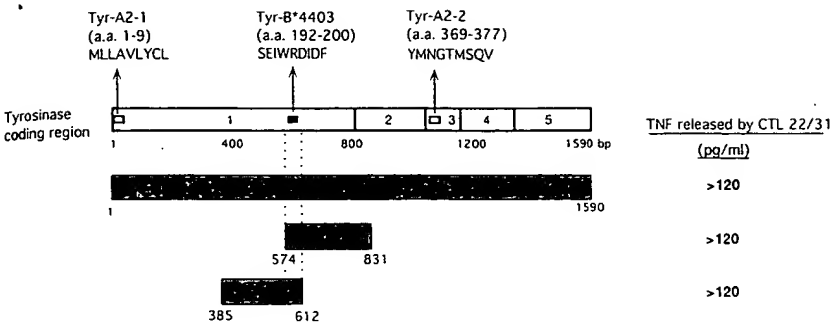

Figure 3. Location of the tyrosinase sequence coding for antigen MZ2-C. The coding part of the cDNA is shown with the exon boundaries. The limits of the cDNA fragments cloned into pcDNAI/Amp and transfected into COS cells together with HLA-B*4403 are numbered from the start of this coding region. The amount of TNF released by CTL 22/31 stimulated with the transfected COS cells is indicated.

with restriction enzymes or by PCR amplification. These fragments were cloned into expression vector pcDNAI/Amp and transfected into COS-7 cells together with HLA-B44. A PCR fragment ranging from nucleotides 574 to 831 conferred the expression of antigen C (Fig. 3). Another PCR fragment ending at position 612 was recognized by CTL 22/31. We concluded that the antigenic peptide was encoded by the sequence located between position 574 and 612. The SEIWRDIDFAHEA peptide encoded by this sequence was synthesized. It sensitized the allogeneic lymphoblastoid cell line LG2-EBV, which expresses HLA-B44, to lysis by CTL 22/31. Shorter peptides were synthesized and tested. The optimal peptide was found to be nonamer SEIWRDIDF (position 192–200) (Fig. 4). Recognition by CTL 22/31 was abolished by removal of the N-terminal Ser (Fig. 4) and also by the replacement of the C-terminal Phe by an Ala (data not shown).

Our experiments were performed using the tyrosinase cDNA clone 123.B2 that we had previously isolated from melanoma cell line SK29-MEL. This cDNA differs in three positions from two other cDNA clones coding for tyrosinase reported by Bouchard et al. [33] and Kwon et al. [34]. One difference is located in the codon coding for the N-terminal Ser residue of nonapeptide SEIWRDIDF. The alternative residue is a Tyr, another uncharged polar amino acid. The allele coding for the Tyr residue is present in ~ 50 % of Caucasians [35, 36]. To test whether this polymorphism could affect recognition by the CTL, we tested nonapeptide YEIWRDIDF. It also sensitized the LG2-EBV target cells to CTL lysis. The peptide concentration required to obtain 50 % of the maximal lysis was close to that required for nonapeptide SEIWRDIDF (data not shown).

3.4 Presentation to CTL 22/31 is restricted to the HLA-B*4403 subtype

Even though our results demonstrated that CTL 22/31 recognized a tyrosinase peptide presented by a HLA-B44 molecule, it was not stimulated by melanoma line SK29-MEL which expressed tyrosinase and was derived from a HLA-B44 patient (Fig. 5) We could exclude that this was due to polymorphism of the tyrosinase gene because the tyrosinase cDNA clone that was used in the experiments described above had been isolated from SK29-MEL.

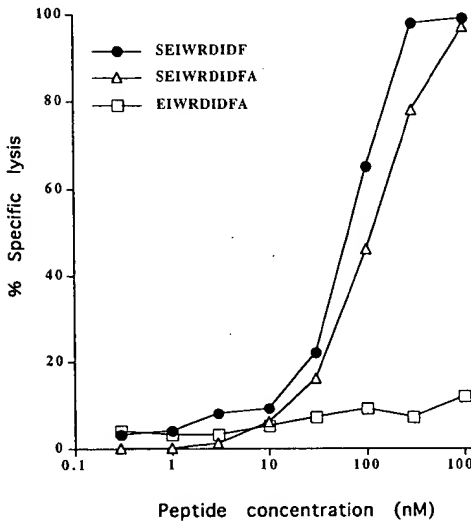

Figure 4. Lysis of target cells incubated with tyrosinase peptides. Lymphoblastoid cells LG2-EBV were Cr-labeled for 50 min. After washing, the cells were incubated for 30 min with the peptides at various concentrations. CTL clone 22/31 was added at an effector to target ratio of 50 and Cr release was measured after 4 h.

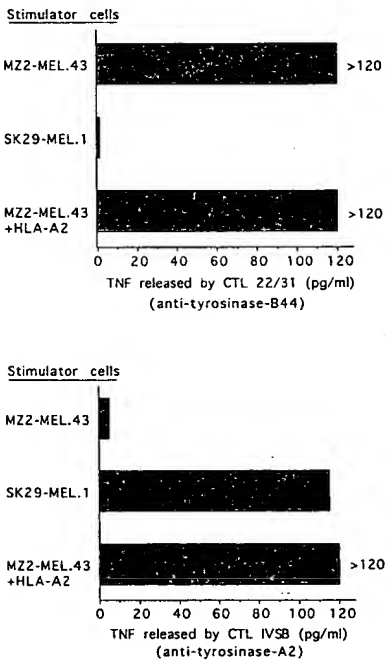

Figure 5. Recognition by CTL 22/31 and CTL IVSB of target cells expressing simultaneously HLA-A2 and B44. MZ2-MEL.43 expresses HLA-B44 only, SK29-MEL.1 is positive for both HLA-A2 and B44. MZ2-MEL.43 + HLA-A2:a MZ2-MEL.43 cell transfected with HLA-A*0201.

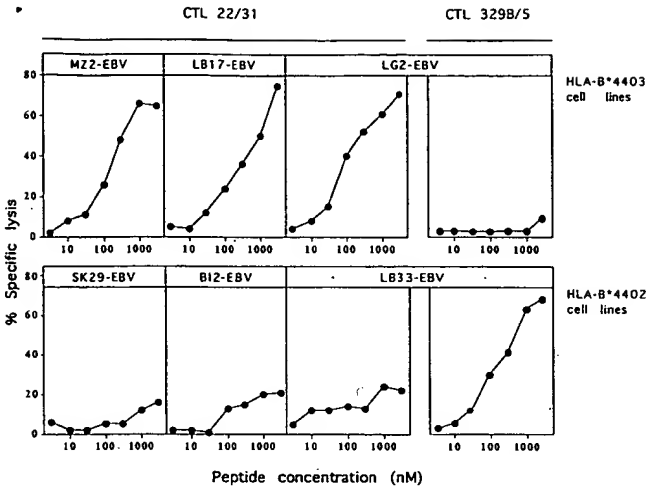

Figure 6. Lysis of lymphoblastoid cell lines expressing the B*4403 or B*4402 subtypes pulsed with the tyrosinase-B44 peptide. Cells were Cr-labeled for 50 min. After washing, they were incubated with peptide SEIWRDIDF for 30 min. CTL clones were added at an effector to target ratio of 30 for CTL 22/31 and 8 for CTL 329B/5. Cr release was measured after 4 h.

We examined the possibility that the presence on SK29-MEL cells of HLA-A2 molecules, which present two tyrosinase peptides, might prevent the other tyrosinase peptide from being presented by HLA-B44 molecules. This was found not to be the case: MZ2-MEL.43 cells transfected with the HLA-A2 gene were still recognized by CTL 22/31 (Fig. 5).

We then tested whether a difference in HLA subtype could account for the absence of recognition of melanoma cell line SK29-MEL. Two major subtypes have been reported for HLA-B44: B*4402 and B*4403. Melanoma cell line SK29-MEL expresses the B*4402 subtype, whereas MZ2-MEL expresses the B*4403 subtype. Lymphoblastoid cell lines of both subtypes were pulsed with nonapeptide SEIWRDIDF and tested for their sensitivity to lysis by anti-MZ2-C CTL 22/31 (Fig. 6). The lymphoblastoid cells that expressed the HLA-B*4403 subtype were lysed very well, whereas little if any lysis was observed on the B*4402 lines. We conclude that recognition by CTL 22/31 is restricted to the B*4403 subtype.

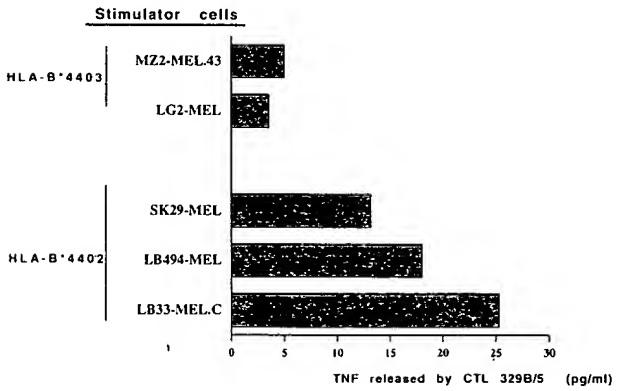

Figure 7. Recognition by CTL 329B/5 of melanoma cell lines expressing the tyrosinase and HLA-B*4402 or -03 subtypes. The cell lines were incubated with CTL 329B/5. One day later, the concentration of TNF in the supernatant was measured.

3.5 Peptide SEIWRDIDF can be recognized by CTL on the HLA-B*4402 molecule

We examined whether the tyrosinase peptide recognized on the B*4403 molecule can also be presented by the B*4402 molecule. The peptide was used as a competitor for a previously defined peptide which is recognized by autologous CTL on a HLA-B*4402 melanoma [37]. The tyrosinase peptide competed efficiently (data not shown), indicating that it binds to HLA-B*4402.

By stimulating $CD8^+$ T lymphocytes of a HLA-B*4402 healthy individual with autologous macrophages and dendritic cells pulsed with peptide SEIWRDIDF, we obtained a CTL population that lysed cells pulsed with the peptide. From this responder population we derived CTL clone 329B/5, that proved able to lyse EBV-transformed cells pulsed with the peptide provided they expressed the HLA-B*4402 subtype (Fig. 6). The ability to stimulate TNF release by CTL 329B/5 was also much higher for B*4402 melanomas than for B*4403 lines (Fig. 7).

4 Discussion

Two peptides encoded by tyrosinase and recognized on HLA-A2 melanoma cells by autologous CTL clones have been described previously [14]. We report here the identification of another tyrosinase peptide which is presented by HLA-B*4403 and forms antigen MZ2-C, which is also recognized by autologous CTL. The peptide corresponds to amino acids 192–200. Other epitopes encoded by tyrosinase are also recognized by autologous tumor-infiltrating lymphocytes (TIL) on HLA-A24 and by $CD4^+$ T cells on HLA-DR4 [38, 39].

A consensus motif for anchor residues binding to HLA-B44 has been recently inferred from the sequence of eluted peptides [40]. The reported motif shows a predominance for Glu at position 2, Tyr or Phe at positions 9 or 10 and hydrophobic residues at position 3. The tyrosinase nonapeptide 192–200 fits this consensus very well with Glu at position 2, Phe at position 9 and Ile at position 3. Other peptides recognized by CTL on HLA-B44 molecules, such as the EBNA3C protein of EBV [41], the p24gag of HIV [42], the nucleocapsid protein of hepatitis C virus [43] and a tumor antigen generated by a point mutation [37] also fit this consensus motif.

Among cells expressing tyrosinase, those of the HLA-B*4403 subtype are lysed by the anti-MZ2-C CTL clone, whereas those of the B*4402 subtype undergo little if any lysis. Such differences between HLA-B*4402 and B*4403 are in agreement with the observation of a strong allograft rejection between a donor and a recipient differing with respect to these two major subtypes of HLA-B44, which differ by a single Asp (B*4402) to Leu (B*4403) substitution in position 156 of the α2 domain [44, 45]. The restriction of anti-MZ2-C CTL by a particular B44 subtype could *a priori* be explained either by the binding of the peptides to only one subtype or by structural differences in the bound peptide induced by differences in the groove of the two subtypes. We observed that the tyrosinase nonapeptide binds to both subtypes. This is consistent with the finding that the consensus motif are similar for both sub-

types B*4402 and B*4403, since consensus motifs appear to be based on anchoring residues [40]. It appears, therefore, that different conformations of the peptide in the B*4402 and -03 grooves account for the difference in the CTL recognition. The substituted residue in position 156, which has been located at the edge of pocket D of HLA-A2, ought to interact with residues 5 or 6 of the bound peptide [46]. The tyrosinase nonapeptide contains in position 5 a positively charged residue (Arg). One can speculate that this residue cannot bind to the hydropobic Leu residue of HLA-B*4403 binding groove, so that it is free to interact with the T cell receptor. In the B*4402 groove, the Arg residue of the peptide could bind to the negative Asp residue, so that it would be buried in the groove. Thus, the T cell receptor would have to fit a different epitope.

The tyrosinase gene is expressed in all melanoma samples [13] and HLA-B44 individuals are very frequent in Caucasian populations (24%) [47]. The polymorphism which affects codon 192 of the tyrosinase gene does not alter CTL recognition of the corresponding peptide. Among the subtypes which have been described for HLA-B44 [45, 48–50], the two major subtypes are HLA-B*4402 (15% of Caucasians) and B*4403 (8%) [49]. Taken together, our data suggest that melanomas expressing tyrosinase and HLA-B44 can be recognized by autologous CTL, in the context of both HLA-B*4402 and B*4403. Therefore, almost all patients typed positive for HLA-B44 could be eligible for pilot studies involving immunization against this tyrosinase epitope. The considerable number of different epitopes encoded by tyrosinase and the frequent occurrence of their presenting HLA molecules make it a good candidate for immunotherapy of melanoma, but the possible negative consequences of over-immunization against tyrosinase, such as destruction of normal melanocytes, remain to be evaluated. Previous observations that antibodies against differentiation protein such as gp75 are found in the sera of some melanoma patients are in line which the notion that differentiation antigens can constitute targets for autologous immune responses [51]. The observation that vitiligo occurring in some melanoma patients is associated with good prognosis [52, 53] suggests that immune responses directed against melanocyte differentiation antigens like tyrosinase may contribute to the elimination of tumor cells in melanoma patients.

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Review

Function of the transport complex TAP in cellular immune recognition

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Abstract

The transporter associated with antigen processing (TAP) is essential for peptide loading onto major histocompatibility complex (MHC) class I molecules by translocating peptides into the endoplasmic reticulum. The MHC-encoded ABC transporter works in concert with the proteasome and MHC class I molecules for the antigen presentation on the cell surface for T cell recognition. TAP forms a heterodimer where each subunit consists of a hydrophilic nucleotide binding domain and a hydrophobic transmembrane domain. The transport mechanism is a multistep process composed of an ATP-independent peptide association step which induces a structural reorganization of the transport complex that may trigger the ATP-driven transport of the peptide into the endoplasmic reticulum lumen. By using combinatorial peptide libraries, the substrate selectivity and the recognition principle of TAP have been elucidated. TAP maximizes the degree of substrate diversity in combination with high substrate affinity. This ABC transporter is also unique as it is closely associated with chaperone-like proteins involved in bonding of the substrate onto MHC molecules. Most interestingly, virus-infected and malignant cells have developed strategies to escape immune surveillance by affecting TAP expression or function.

Keywords: ABC transporter; Transporter associated with antigen processing; Antigen presentation; Transport mechanism; Virus persistence

Contents

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1. Introduction – overview of the MHC class I antigen processing pathway

Under normal conditions class I molecules of the major histocompatibility complex (MHC) present peptides derived from endogenous proteins on the cell surface of every nucleated cell (Fig. 1). During viral infection or malignant transformation, an additional set of peptides bound to MHC class I molecules is delivered to the cell surface for presentation to cytotoxic T lymphocytes (CTL). The recognition of MHC class I molecules ('self-component') loaded with peptides derived from 'non-self' proteins by CTL via the T cell receptors eventually leads to the lysis/apoptosis of abnormal cells (for review see [1–4]).

The pathway of antigen presentation on the cell surface of pathogens dwelling in the cytosol comprises proteins from different compartments. The pathogens together with their intrinsic proteins are degraded by the major cytosolic proteolysis machinery, the 20S/26S proteasome (for review see [5–8]). After cleavage, the peptides are translocated by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER) where assembly of MHC class I molecules and peptides occurs. Stable ternary complexes consisting of heavy chain, β_2-microglobulin (β_2-m) and bound peptide can leave the ER for surface presentation to T cell receptor.

Every human has three to six different MHC class I alleles for the presentation of antigens on the cell surface. This low number of different MHC class I molecules has to bind peptides of every non-self protein for presentation to CTL and protection of the individuals against pathogens. As revealed by X-ray crystallography, the antigenic peptides bind in a groove formed by two α-helices on the rim and eight β-strands on the bottom containing residues of the α1 and α2 domains of the heavy chain of MHC class I molecules [9–11]. The peptides are fixed via the free amino- and carboxy-termini. In addition, anchor residues at position two or three and at the carboxy-terminal residue pointing into the groove are important. In between these anchor residues, the side chains point outside the groove which explains the large pool of peptides that can be presented by one MHC class I allele. Interestingly, the T-cell receptor monitors this variable region [12,13]. The groove is closed on both ends, therefore the size of the peptides is determined to 8–10 residues in length.

The assembly of MHC class I molecules has been well studied (for review see [14–16]). However, less is known about how the peptides are generated and transported into the ER [4,17]. The contribution of the 20S proteasome for the generation of antigenic peptides came from the observation that the two β-type proteasomal subunits, LMP2 and LMP7 (low molecular weight peptides), were identified within the MHC locus [18–20]. The proteasome complex is a multicatalytic macromolecular protease (700 kDa) consisting of 28 subunits arranged in two outer rings of α-type and two inner rings of β-type subunits (for review see [5,7]). LMP2 and LMP7 are upregulated by interferon-γ (INF-γ), which causes a structural rearrangement of the 20S proteasome in which the catalytically active subunits β2, β1 and β5 are replaced by MECL1 (multicatalytic endopeptidase complex-like), LMP2 and LMP7, respectively, and the so-called immunoproteasomes are formed. Contradictory results exist about the functional difference and cleavage specificity of the immunoproteasome compared to the proteasome consisting of constitutively expressed β-type subunits. Due to the substitution of the active subunits, the enzymatic activity of the immunoproteasome cleaving after hydrophobic and basic substrates is increased whereas the peptidyl-glutamyl activity is decreased in comparison to the housekeeping proteasome [21–24]. This alteration may fit with the preference of MHC class I molecules for peptides with hydrophobic and basic carboxy-terminal peptide residues. The peptides produced by the proteasome have a size distribution of approximately 3–30 residues with a maximum of 6–11 residues [21,25–27] which are also in part the size of the antigenic peptides bound in the groove of the binding pocket of MHC class I molecules. In addition to the proteasome, other proteases such as an IFN-γ-inducible leucine aminopeptidase [28] or a giant cytosolic protease system in the cytosol [29,30] may play a role in epitope generation. Vinitsky and coworkers showed that the presentation of antigenic peptides of influenza viral proteins is not influenced by blocking proteasomal activity [26]. In addition, epitopes may also be produced by proteolytic trimming in the ER after TAP-dependent transport [31].

The important role for peptide transport into the

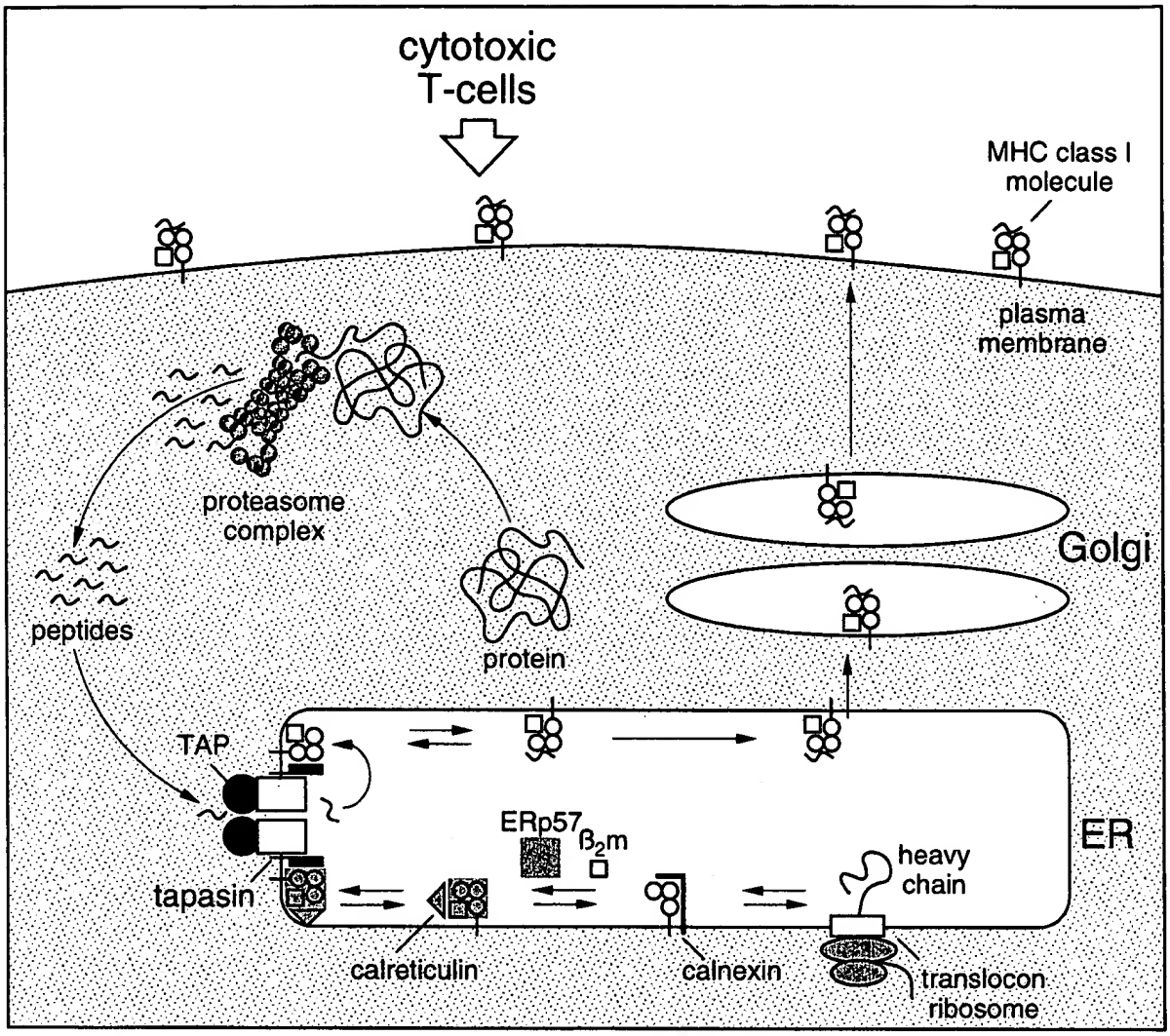

Fig. 1. Antigen processing and presentation via MHC class I molecules. Endogenous proteins including viral or tumor-specific proteins are degraded in the ubiquitin-proteasome pathway, and peptides are transported into the ER lumen by TAP. There, several molecules have been implicated in the tightly regulated folding, assembly and loading of MHC class I molecules, including calnexin, calreticulin, tapasin, ERp57, and possibly a peptide trimming activity. Stable MHC-peptide complexes can leave the ER via the Golgi compartment to the cell surface for recognition by cytotoxic T lymphocytes.

ER lumen became clear from studies of various cell lines with a strongly reduced level of MHC class I molecules on the cell surface [32,33]. Although the expression levels of MHC class I heavy chain and β_2-m are normal and exogenously added peptides or peptides introduced in the ER by a signal sequence were efficiently presented, these defective cell lines were unable to present intracellular antigens on the cell surface. The defective phenotypes could be restored by transfection of *tap1* and/or *tap2* [34,35]. The genes for human TAP1 and TAP2 are located in the MHC II locus of chromosome 6 and comprise 10 kb each. The genes are encoded by 11 exons. Eight exons have the same size and all exon/intron boundaries are identical [36]. All four genes are organized in an array *lmp2*, *tap1*, *lmp7*, *tap2*, where only *lmp2* is encoded on the (−) strand, suggesting a duplication of an ancestral gene for a pro-

teasomal β type and TAP subunit followed by an inversion of *lmp2*. Transcription of all four genes is induced by IFN-γ, indicating a common regulation and concerted function of these genes in antigen processing.

By using isolated microsomes or semi-permeabilized cells, ATP- and TAP-dependent peptide translocation into the ER was demonstrated [37–39]. In addition, expression of TAP in insect cells and yeast revealed that TAP is functional in the absence of factors of the adaptive immune system [40,41]. In addition to this major TAP-dependent translocation mechanism, alternative, TAP-independent pathways may exist for processing and loading of peptides onto MHC class I molecules [42].

The TAP sequences of human, gorilla (*Gorilla gorilla*), mouse (*Mus musculus/castaneus*), rat (*Rattus norvegicus*), hamster (*Mesocricetus auratus*), and salmon (*Salmo salar*) show phylogenetic differences as expected. Human TAP1 possesses 98.8% homology with gorilla TAP1, 69.2% with hamster TAP1 and only 40% with salmon TAP1. The homology between TAP1 and TAP2 in all species is approximately 35% although they have a similar predicted membrane topology. Thus, TAP1 and TAP2 seem to be derived from an ancestral gene by gene duplication that happened before the evolution of the adaptive immune system found in vertebrates.

The *tap* genes of all species examined are polymorphic, but only the rat alleles [43] as well as the most recently discovered human TAP2iso splice variant [44] possess different substrate specificity. The cim (class I modifier) polymorphism in rat is based on four tap2 alleles grouped into cim^a and cim^b [43]. Cim^b comprises the b and u alleles with a specificity for hydrophobic, carboxy-terminal residues of the processing peptide [45,46]. In contrast, the haplotypes of cim^a (a and l) show a much broader specificity and can transport peptides with basic or hydrophobic residues at the carboxy-terminus. The cim^a and cim^b alleles differ in 25 residues whereas only two of them are located in the nucleotide binding domain of TAP2. The remaining divergent residues are in the amino-terminal half of the subunits. For human or murine TAP approximately 10 polymorphic sites have been found for each subunit. However, these alleles do not show any difference in substrate specificity [47,48].

2. Structural organization of the TAP complex

TAP1 and TAP2 belong to the superfamily of ATP-binding cassette (ABC) transporter that comprises a large number of polytopic integral membrane proteins transporting a diverse set of molecules across membranes in an ATP-dependent manner [49,50]. ABC transporters are found in all three domains of life representing in some organisms the largest family of paralogous proteins. All ABC transporters possess two conserved cytoplasmic ATP-binding domains and two hydrophobic domains comprising 5–10 transmembrane stretches, possibly α-helices, lining up the putative translocation pore. Although the structural organization of the ABC transporters can be assumed to be very similar, the hydrophobic transmembrane domains show only very weak sequence homology. TAP1 or TAP2 are so-called half-size transporters composed of a hydrophobic transmembrane domain (TMD) followed by a highly conserved nucleotide binding domain (NBD). There is 60% sequence identity within the NBDs and only 30% between the TMDs of TAP.

Human TAP1 and TAP2 have a length of 748 amino acids (81 kDa) and 686 amino acids (75 kDa), respectively. The molecular weight determined experimentally by SDS-polyacrylamide gel electrophoresis is 71 kDa for TAP1 and 75 kDa for TAP2. Both proteins are located in the ER and *cis*-Golgi [51] retarded by a so far unknown cryptic ER retention signal. For human TAP2, an allele that is 17 amino acids longer (703 aa) has been identified resulting from a polymorphic single base pair substitution in the stop codon of the shorter TAP2 allele [52]. Recently, a splice variant of hTAP2 with a length of only 653 amino acids was detected [44]. As also found for other ABC transporters synthesized at the ER, both TAP proteins lack an N-terminal signal sequence for the import in the ER, suggesting that an internal signal sequence may exist promoting insertion in the ER membrane. TAP1 and TAP2 are found to be non-glycosylated although human TAP1 has three consensus glycosylation sites, two facing the cytosol and one placed in a short ER loop which are too short for effective glycosylation by the oligosaccharide transferase [40]. A very minor subpopulation of hTAP was found to be *N*-glycosylated [53] which may reflect misfolded protein.

By immuno-coprecipitation it was shown that TAP1 and TAP2 assemble in the ER membrane to form a heteromeric complex [54,55]. Coexpression of TAP1 and TAP2 in TAP-deficient cell lines demonstrated that both subunits are essential for antigen processing [34,35,56]. Furthermore, no additional factors of the immune system are required for TAP function [40,41]. Gel filtration analysis together with more recent crosslinking experiments suggested that TAP1 and TAP2 form a functional heterodimer within the ER membrane with a stoichiometry of 1:1 [40,57,58].

As mentioned, the homology between the hydrophobic domains of ABC transporters is not very high and so there is a variability in the number of transmembrane domains. Six transmembrane helices are predicted for hemolysin transporter HlyB [59,60]. The subunits MalG and MalF of the maltose transporter are supposed to contain six and eight transmembrane helices, respectively [61,62]. Some eukaryotic ABC transporters show six transmembrane helices of each hydrophobic domain with the amino- and carboxy-terminus in the cytosol. Such a 2×6 transmembrane helix model could also be confirmed for MDR1 [63,64]. As predicted from hydrophobicity analysis, the membrane topology of human TAP1 and TAP2 is supposed to be different (Fig. 2) [65]. Sequence alignments with other ABC transporters exclude the first 175 residues for hTAP1 and 140 residues for hTAP2. This part of TAP (N-domain) displays no sequence homology with any protein. In addition, the N-domain is very hydrophobic comprising four and three predicted transmembrane helices for TAP1 and TAP2, respectively. Although no function of this amino-terminal hydrophobic region is known so far, it can be speculated that it is essential for ER membrane targeting and correct assembly of the complex. The rest of the transmembrane domain shows weak, but significant sequence homology to MDR1 and other ABC transporters of the P-glycoprotein/TAP subfamily. The sequence similarity is increased from TM1 to TM6. Based on the hydrophobicity analysis and sequence alignments with MDR1 for which the membrane topology has been extensively studied, a 2×6 transmembrane helix model of TAP can be derived extended by an additional four and three transmembrane helices predicted for the N-domain [65]. Interestingly, the TAP complex is highly asymmetric and only a very small portion (< 10%) is in the ER lumen, whereas large cytosolic loops and both NBDs are in the cytosol.

In a first experimental approach, 10 transmembrane helices were reported for hTAP1 expressed in *Escherichia coli* [66]. The topology was studied using carboxy-terminal truncations of hTAP1 fused to β-lactamase as reporter. This approach has been quite successful in addressing the topology of several membrane proteins of prokaryotes. However, care must be taken if multisubunit ABC transporters from highly specialized eukaryotic intracellular compartments are studied. The topology model of hTAP1 found in this study contradicts the orientation of the loop between TM5 and TM6 that has been identified by peptide photocrosslinking experiments to be involved in peptide binding from the cytosol [67,68]. Differences in membrane topology were also reported for P-glycoprotein expressed in *E. coli* [64] in comparison to the expression in eukaryotic cells [69]. Notably, TAP1 expressed in *E. coli* is not functional as large carboxy-terminal regions and the entire TAP2 protein are missing, both are likely to be essential for correct membrane insertion. It has been further shown that even the insertion of an epitope, a glycosylation targeting sequence or a protease cleavage site into transmembrane loops of ABC transporters resulted in a loss of protein function and possibly incorrect folding into the membrane. Thus, alternative methods have to be applied to address the topology of the TAP complex. Following the elegant approach reported for lactose permease [70] and MDR1 [69,71], it will be interesting to construct cysteine-less mutants of TAP1 and TAP2 that are functional. Subsequently, single cysteines can be introduced in predicted loops and their accessibility can be probed by thiol-specific reagents. Mutation of all 19 cysteines of TAP is very time-consuming, but these cysteine mutations are supposed to be minimally invasive to the structure and function of TAP.

In TAP, the hydrophobic transmembrane domains are linked to the nucleotide binding domains which harbor the highly conserved ATP-binding cassette consisting of the Walker A and B motifs (A/B) for ATP binding and hydrolysis [72]. All ABC transporters have a so-called C-loop (C) that comprises six to eight conserved amino acids which are located sev-

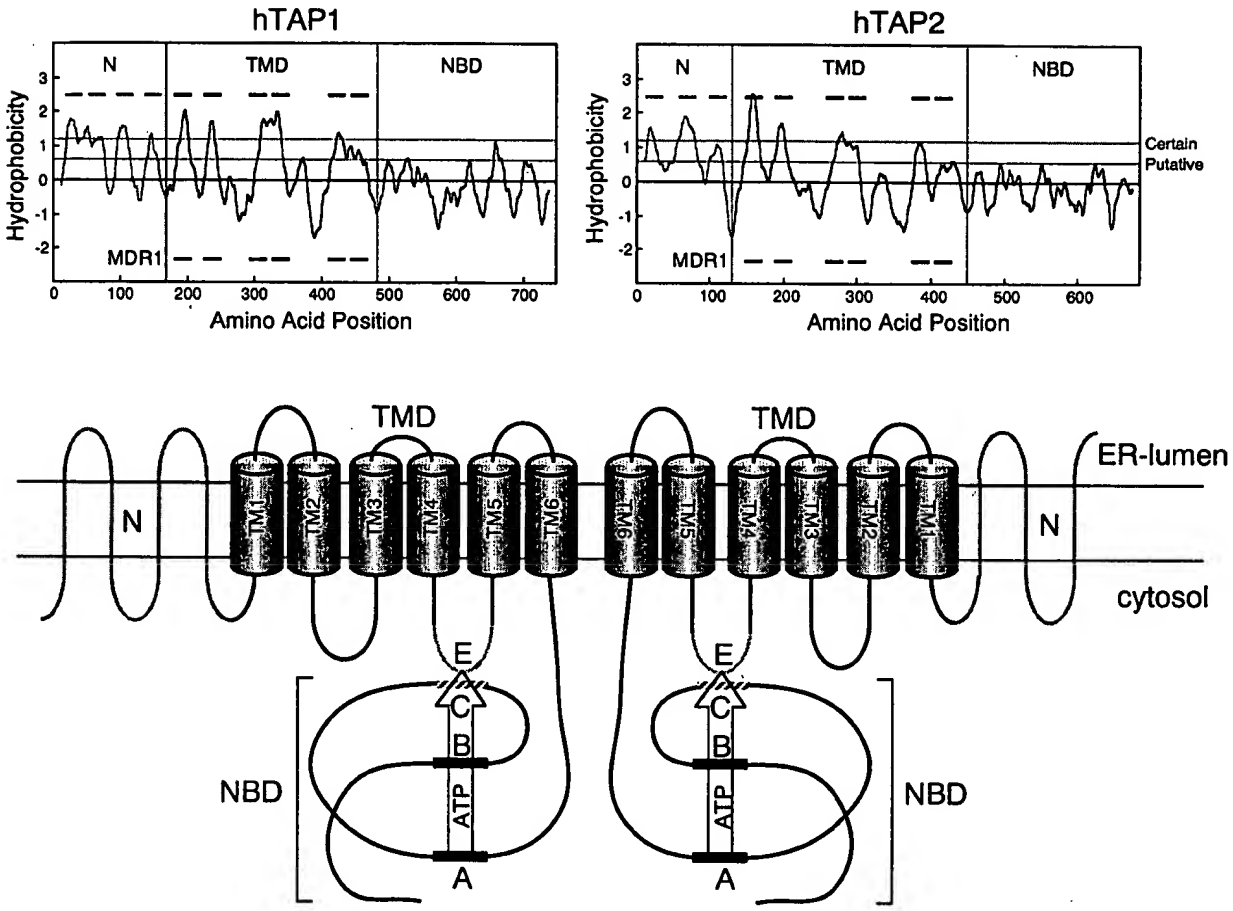

Fig. 2. Membrane topology of the human TAP complex. The membrane topology was predicted by sequence alignment with human MDR1 and using TopPredII (M.-G. Claros, Paris, France, based on the algorithm of Gunnar von Heijne). For the hydrophobicity plot the following parameters were used (Kyte-Doolittle; full window: 21; core window: 11). From the hydrophobicity plot four and three transmembrane helices can be predicted for the hydrophobic N-terminal domain (N) of TAP1 and TAP2, respectively. The communication between the NBD and TMD leads to tight coupling of peptide binding and ATP hydrolysis probably via contact of the C-loop (C) and the 'EAA'-like region (E). The Walker A (A) and B (B) regions fix the ATP at the nucleotide binding pocket.

eral residues upstream of the Walker B sequence. The 'EAA'-like motif (E) found in the last cytosolic loop of the membrane spanning domain of bacterial ABC transporters [73] is less significant for TAP. There is genetic evidence that this 'EAA' sequence interacts via the C-loop with the nucleotide binding domain and couples ATP-hydrolysis to the transmembrane spanning domain [74,75].

Peptides are photocrosslinked to TAP1 and TAP2 suggesting that both subunits contribute to peptide binding [76,77]. In more recent studies, the peptide binding site was mapped to regions of human TAP1 and TAP2. The crosslinked subunits were digested by trypsin and/or bromocyan and crosslinked fragments were immunoprecipitated with antibodies against epitopes of putative loops in TMD [67,68]. The proteolytic analysis of the crosslinked TAP subunits revealed a similar binding region for TAP1 and TAP2. The binding region comprises the cytosolic loop between putative TM4 and TM5 and a carboxy-terminal stretch of approximately 15 amino acids following TM6. Due to the topological model (Fig. 2), all these binding regions are exposed to the cytosol. Regarding the transmembrane helices, TM4 to TM6 seem to be involved in peptide binding. For TAP2, the ER-exposed loop between predicted TM1 and

TM2 is also implicated in peptide binding [67]. The identified photocrosslinked regions also comprise the polymorphic residues 374 and 380 of rat TAP2 which alter the substrate specificity in the rat TAP2^a and TAP2^u alleles [78,79]. In future, a more detailed analysis of the substrate binding site may also become possible using single cysteine mutants as described above.

To obtain information about which subunit of the TAP complex is involved in substrate specificity, Armandola and coworkers coexpressed TAP subunits from different species and tested their substrate specificity using peptides with different carboxy-terminal residues [80]. By using chimeric rTAP2^a or rTAP2^u and hTAP2, residues located between residues 1 and 361 of hTAP2 were identified to affect the specificity for peptides with various carboxy-terminal residues. Moreover, two pairs of residues (217/218) and (374/380) of rat TAP and a single point mutation (A374D) of human TAP affect the substrate specificity [78,80]. Taking these data together, transport specificity with regard to the carboxy-terminal residue of the peptide substrate is mainly affected by TAP2, but TAP1 can also contribute. The influence of TAP2 on peptide binding seems to be more complex because TAP2iso resembling a truncated splice variant of hTAP2 showed an altered peptide specificity [44]. Thus, in addition of regions described above, the last 33–51 carboxy-terminal amino acids of TAP2 seem also to contribute to peptide specificity. How this carboxy-terminal region of TAP2 affects the peptide selection remains an open and puzzling question.

Similar to P-glycoprotein [81,82], the region of TM5 and TM6 of both subunits seems to build the substrate binding site (Fig. 2). Less is known about the peptide translocation pathway, but it can be speculated that TM5 and TM6 are also part of a translocation channel. Most strikingly, the putative pore seems to have an extended diameter because peptides with very large side chains such as fluorophores or octapeptides can be efficiently bound and transported by TAP [83–85]. Interestingly, peptides labeled with gold clusters of 1.4 nm in diameter bind specifically to TAP (L. Neumann and R. Tampé, unpublished results). Moreover, some peptides containing hydrophobic side groups have higher affinity for TAP than the unlabelled peptide [83,84].

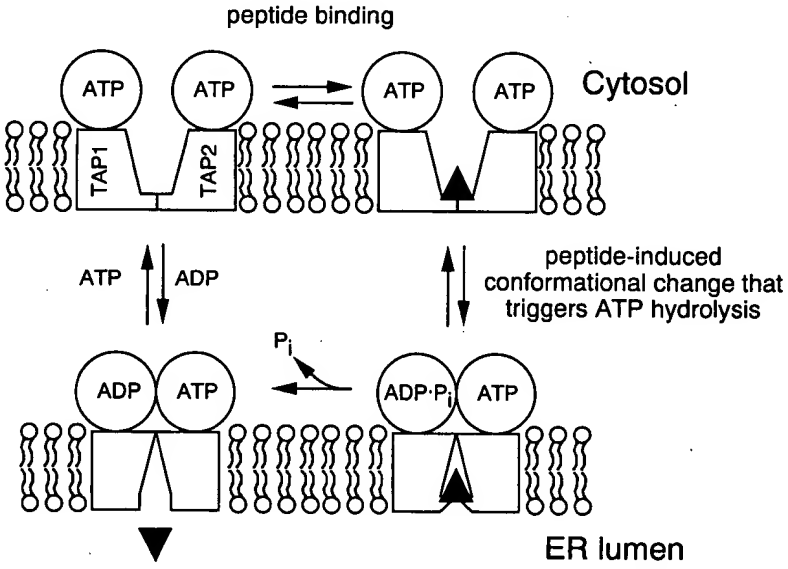

Fig. 3. Model of substrate binding and translocation by the TAP complex. Peptide (▲) and ATP bind to TAP from the cytosol in an uncoordinated event. Under physiological conditions, it can be assumed that the NBDs are already loaded with ATP/ADP. Peptide binding to TAP induces a structural reorganization of the TAP-peptide complex triggering ATP hydrolysis in the NBD which subsequently leads to the transport of the peptide across the ER membrane and the release in the ER lumen. However, we cannot distinguish whether ATP hydrolysis at both NBDs is required for substrate translocation (parallel mode) or whether ATP hydrolysis at one NBD drives the translocation leaving the second NBD for completion of the transport cycle (sequential mode).

3. Multistep transport mechanism of TAP

Peptide transport by TAP is a multistep process (Fig. 3). Peptide associates with TAP in an ATP-independent manner following a monophasic 1:1 Langmuir binding model (A+B↔AB) [83,86]. In direct peptide binding or competition assays as well as photocrosslinking experiments, no indication for a second binding site was found. However, it cannot be entirely ruled out that a second binding site with very similar affinity or alternatively with very low affinity exists. By using peptides labeled with an environmentally sensitive fluorophore, the association pathway to TAP could be kinetically dissected in real time [84]. Peptide binding to TAP is composed of a fast bimolecular association step followed by a slow isomerization of the TAP-peptide complex. This structural reorganization may trigger the ATP hydrolysis and substrate translocation across the membrane. TAP selectivity is primarily determined by the first bimolecular association step. Further evidence for a structural isomerization of the TAP complex was deduced from chemical crosslinking experiments in which the level of crosslinked TAP heterodimers increased in the presence of peptide [57].

Peptide translocation strictly requires the hydrolysis of Mg·ATP [37–40]. Non-hydrolyzable ATP analogs, such as ATPγS, AMP-PNP or AMP-PCP, do not promote peptide transport. Peptide transport can be energized by ATP, UTP, CTP and GTP [39,87]. The Michaelis-Menten constant K_M (Mg·ATP) for peptide transport is 0.1–1 mM [87]. Direct binding of nucleotides was demonstrated by 8-azido-ATP photocrosslinking experiments [53,88,89]. Interestingly, ATP and ADP have similar affinity for TAP, explaining that peptide transport can be inhibited by ADP and other nucleoside diphosphates competing for ATP binding [87]. Both nucleotide binding domains interact with ATP even if separately expressed [88,89]. However, the NBDs of TAP1 and TAP2 are unable to hydrolyze ATP, suggesting that ATP hydrolysis requires a certain arrangement of both NBDs via the transmembrane domains mediated by peptide binding. In addition, both NBDs are essential for TAP function because mutation of one NBD (R659Q of hTAP1) leads to a loss of transport function [90].

By partial purification and reconstitution into proteoliposomes, the ATPase activity of the TAP complex could be analyzed for the first time (S. Gorbulev and R. Tampé, manuscript in preparation). The ATPase activity is substrate-specific and can be blocked by a viral TAP inhibitor (see Section 5). Most strikingly, the ATPase activity is tightly coupled to peptide binding, indicating that substrate binding is a requisite step for subsequent ATP hydrolysis. Thus, peptide binding may cause a structural rearrangement of the NBDs that could function as a molecular switch to activate the ATPase of TAP thereby preventing the waste of ATP without translocation of peptides.

Regarding the final ATP-dependent translocation step, important questions remain open. Do the two NBDs work in sequential or synchronous fashion which implies the question whether one or two ATP are needed for a complete transport cycle? Furthermore, are both NBDs equal in function, or does ATP hydrolysis at one NBD drive the peptide translocation whereas hydrolysis at the other is needed for completion of the transport cycle and reconversion of the initial substrate binding site exposed to the cytosol? It will be interesting to see in the future how closely TAP resembles transport models of MDR or CFTR (for review see [91]).

4. The substrate binding motif of TAP – coevolution of affinity, specificity and diversity

In contrast to our limited understanding of the structure of TAP including the spatial arrangement of the substrate binding pocket, the substrate specificity of TAP has been well studied. The first generation of results came from experiments based on trapping transported peptides in the ER via glycosylation. Comparing the number of glycosylated peptides differing in amino acid composition or length, information about sequence and length preferences of the transported peptides was extracted (for review see [4,92,93]). The most efficient transport was observed for peptides with a length of 8–12 amino acids [94] whereas van Endert et al. [86] showed an optimum of peptides with 8–16 amino acids for peptide binding. However, peptides of six or 40 amino acids in length are also transported with TAP but with lower efficiency as compared to peptides with 8–12

amino acids. In conclusion, TAP preferentially transports peptides similar or slightly larger in length suitable for MHC class I binding. In an alternative approach, TAP selectivity was studied by ATP-independent peptide binding assays [83,86]. The specificity studied with both methods showed the same affinity pattern for different substrates, but with notable differences in absolute values of the affinity for TAP [76,83,95]. The glycosylation method may lead to an underestimation of differences of transported peptides because this assay includes several side reactions such as transport, glycosylation, degradation and peptide export. Each step has its own kinetic parameter and specificity. In contrast, peptide binding to TAP is a simple bimolecular reaction. Thus, the binding assay seems to be more accurate in resolving subtle details of the binding motif than the glycosylation assay.

To resolve the recognition principle and the substrate binding motif of TAP, complex peptide libraries were applied [96]. With this combinatorial method comparing the average affinity of a randomized peptide mixture with one residue in common with a totally randomized peptide mixture, it was possible to determine the influence on the affinity to TAP of each peptide residue independently of a given sequence context. Thus, the effect of each amino acid residue on stabilization of peptide binding to TAP could be determined. The peptide with the highest affinity showed a 45-fold higher affinity than a totally randomized peptide mixture resembling the selectivity of TAP for certain peptides. In a similar approach using an octapeptide library, a murine MHC class I allele showed a 200-fold higher affinity for a high affinity peptide [97]. On the basis of this coarse comparison, TAP seems to be less restrictive than MHC class I molecules. Most interestingly, the effect of amino acids on the binding to TAP is critically dependent on the position in the nonapeptide. As reported earlier, the strongest differences in peptide binding affinity were observed at the carboxy-terminus of the peptides, as seen for some rat and mouse alleles [45,98]. Human TAP showed a preference for peptides with hydrophobic or basic amino acids (Phe, Leu, Arg or Tyr) at the carboxy-terminus which are also preferred peptide anchors for MHC class I binding (Fig. 4). It should be mentioned that none of the disfavored residues Asp, Glu, Asn or Ser

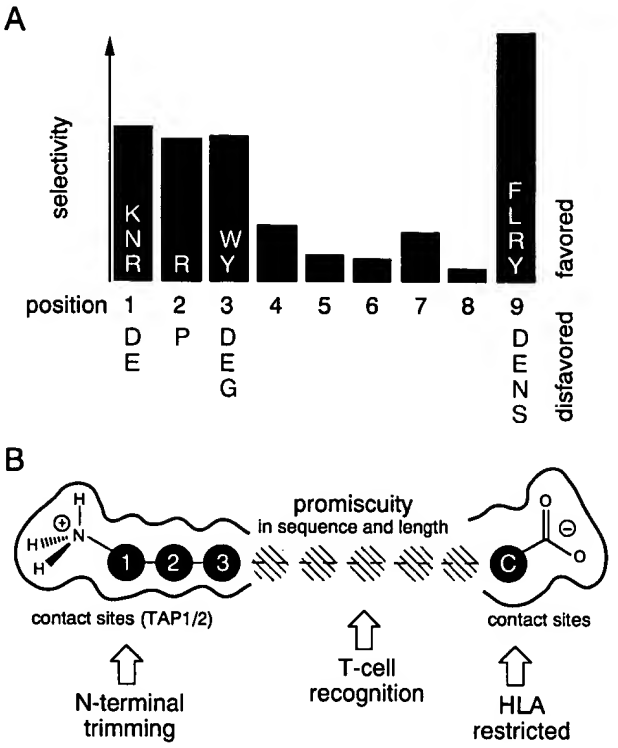

Fig. 4. Substrate recognition motif and substrate binding pocket of human TAP. TAP selectivity is illustrated for positions of the peptide. Favored (white) and disfavored residues (black) of TAP are given at the individual positions as extracted using combinatorial peptide libraries [96]. A model of the substrate binding pocket is shown in the lower panel.

at the carboxy-terminus are anchors for peptide binding to MHC class I molecules. Thus it can be speculated that the recognition principle of TAP and MHC class I molecules coevolved to transport preferentially peptides which later on are bound to MHC class I molecules. Moreover, there are hints that the proteasomal subunits LMP2 and LMP7 upregulated by INF-γ upon immune stimulation increase the tryptic and chymotryptic activity of the proteasome while reducing the peptidyl-glutamyl-peptide hydrolyzing activity [99]. Thus more peptides available for MHC class I binding are generated. The amino-terminal residues of peptides binding to TAP and to MHC class I molecules do not agree very well. Human TAP favors Arg at the second peptide position although Arg is only a weak anchor residue for certain MHC class I alleles at this position. In contrast, Pro at position 2, reducing the affinity for TAP dras-

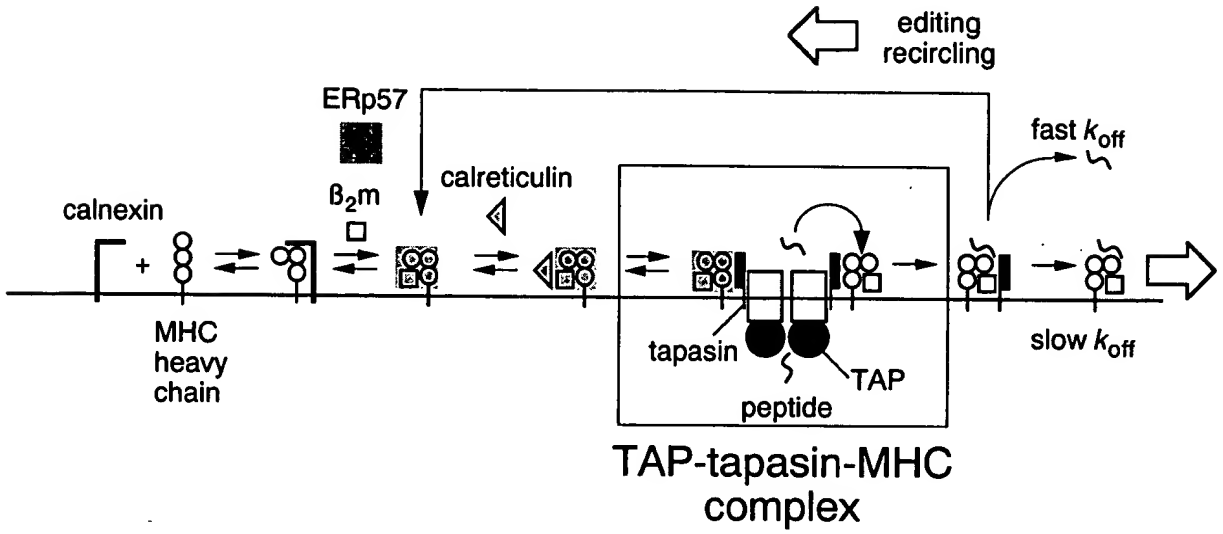

Fig. 5. Pathway of assembly of MHC class I molecules and formation of the macromolecular TAP-tapasin-MHC complex critical for loading of antigenic peptides onto MHC class I molecules. Peptide-loaded MHC molecules can dissociate from the TAP complex. Kinetically unstable MHC-peptide complexes (fast k_{off}) are trapped and recycled by association with tapasin and TAP. Only kinetically stable MHC complexes (slow k_{off})leave the ER via the Golgi to the cell surface ('quality control mechanism').

tically, is the preferred amino acid for other alleles at this position. A similar effect for Pro at position 3 was found for murine TAP [100] suggesting that it is an important principle. Supported by a series of indications, it can be speculated that an amino-terminal exopeptidase located in the ER lumen processes the peptides transported by TAP for loading of some epitopes. This model is supported by the fact that TAP transports peptides that are longer than those bound to MHC class I molecules. While MHC class I alleles use positions 2 and 3 as anchors at the amino-terminus, position 1 of the peptide substrate is also important for TAP selectivity. Here, Lys, Asn and Arg are favored, whereas Asp and Glu weaken TAP binding.

To determine the contribution of the peptide backbone and steric constraints to the peptide binding to TAP, substrates with different lengths were modified by D-amino acids in each position and tested for TAP binding. Only D-amino acids in positions 1–3 and the carboxy-terminal position showed a strong decrease in affinity. This result together with the effect of proline in position 2 supports the idea that the peptides are fixed at positions 1, 2 and 3 via contacts mostly to the peptide backbone. The carboxy-terminus of the peptide functions as a second important anchor selecting for peptides with hydrophobic and basic amino acids. Most importantly, the peptides are fixed at the free amino- and the carboxy-terminus via hydrogen bonding [96]. In analogy to MHC class I molecules, it seems likely that the amino- and carboxy-termini contribute largely to the free binding enthalpy [101]. In conclusion, residues and modifications of the peptide within the amino- and carboxy-terminal anchor regions do not influence the binding and transport by TAP, indicating that this part of the peptide does not interact with TAP (Fig. 4, lower panel). This binding motif explains that longer peptides can bulge out of the substrate binding pocket and that modifications by very large amino acid side chains are tolerated within this region. Most strikingly, the T cell receptors make contacts mainly between residues 5–8 of MHC class I-associated peptides [12,13]. Thus, the T cell recognition takes place in an area where TAP shows the lowest specificity, therefore enlarging the pool of peptides presented on MHC class I molecules. Thus, the proteasome, TAP and MHC class I molecules might have co-evolved a similar substrate specificity to optimize the antigen processing machinery.

5. TAP as part of a macromolecular transport and chaperone complex

In the assembly and loading of MHC class I molecules a growing number of auxiliary, chaperone-like proteins has been identified (for review see [102,103]). Some of them are in close association with TAP (Fig. 5). Calnexin and calreticulin bind to monoglycosylated core glycans in the ER lumen and increase the efficiency of correct folding of glycoproteins (see review [104]). When the binding of these chaperones is inhibited, for example by glucosidase inhibitors, the folding and subsequent surface expression of MHC class I molecules is drastically decreased [105,106]. Calnexin, a type I membrane protein, binds to nascent MHC class I heavy chain before assembly with β_2-m and correct folding occur. The thiol-dependent reductase ERp57 found in complex with MHC class I heavy chain, calnexin and calreticulin also binds in a very early step of the maturation probably supporting the correct formation of the conserved disulfide bridges [107–109]. Then calreticulin, a soluble protein with high sequence homology to calnexin, binds to MHC class I heavy chain perhaps displacing calnexin and supporting the assembly of heavy chain and β_2-m. Furthermore, calreticulin seems to retain empty MHC class I molecules in the ER. Most strikingly, MHC class I molecules are found in close association with the TAP complex [110,111]. As reported by Powis, MHC class I molecules interact with TAP1 and TAP2 [112]. Approximately four MHC class I molecules seem to be linked via four tapasins to one TAP complex [58]. Tapasin is a ER-resident type I glycoprotein consisting of two immunoglobulin folds [58,113]. The reason for this unexpected stoichiometry could be the presence of different MHC class I alleles to increase the efficiency of antigen processing and presentation. In addition to its critical role in mediating the TAP-MHC association, tapasin also has an important function in the assembly and peptide loading of MHC class I molecules probably based on a chaperone effect. As shown by Lehner and coworkers, truncated tapasin missing the transmembrane region and the cytosolic tail does not associate with the TAP complex but still rescues MHC class I surface expression [114]. Finally, tapasin increases the level of TAP [114]. It is not unlikely that more, at the moment unknown proteins are involved in the assembly and loading of MHC class I molecules. In addition to the thiol-dependent reductase activity, ERp57 also has a cysteine protease activity that may possibly process the TAP-derived peptides for optimal binding to MHC class I molecules [107].

6. Implication in human diseases

In the past few years, it has become evident that TAP is involved in several human diseases. Herpes- and adenoviruses are known to interfere with antigen presentation by a downregulation of MHC class I molecules on the cell surface of virus-infected cells (for review see [115]). The immediate early gene product ICP47 of herpes simplex virus type 1 (HSV-1) was identified to inhibit peptide loading onto MHC class I molecules thereby evading detection by cytotoxic T lymphocytes [116]. ICP47 inhibits peptide translocation into the ER [117,118] by blocking the peptide binding site of TAP with high affinity [119,120]. ICP47 is highly species-specific because the affinity for mouse TAP is 100-fold reduced in comparison to human TAP [119,120]. For the efficient inhibition of peptide binding a 32 amino acid fragment comprising amino acid 3–34 of ICP47 is sufficient as seen by binding studies with truncated ICP47 [121]. Which part of the 32mer associates with the peptide binding site is not known so far. However, a photocrosslinker positioned on the amino-terminus of the ICP47 fragment labels both subunits to the same extent whereas ^{125}I-labeled Tyr21 polypeptide crosslinks only TAP1, suggesting that ICP47 is asymmetrically bound to TAP [122]. Upon binding to membranes, ICP47 seems to undergo a conformational change from a loosely folded to an α-helical structure because structural studies showed that the 32mer is loosely structured in aqueous solution. However, by binding to membranes, the polypeptide seems to adopt an α-helical structure [123]. The structure of the active domain of ICP47 was analyzed by NMR. In detergent solution, the active domain of ICP47 consists of two α-helices at residues 3–13 and 23–32 connected by a flexible loop [124].

Human cytomegalovirus encodes at least four different proteins that inhibit the cell surface expression of MHC class I molecules in different ways and at

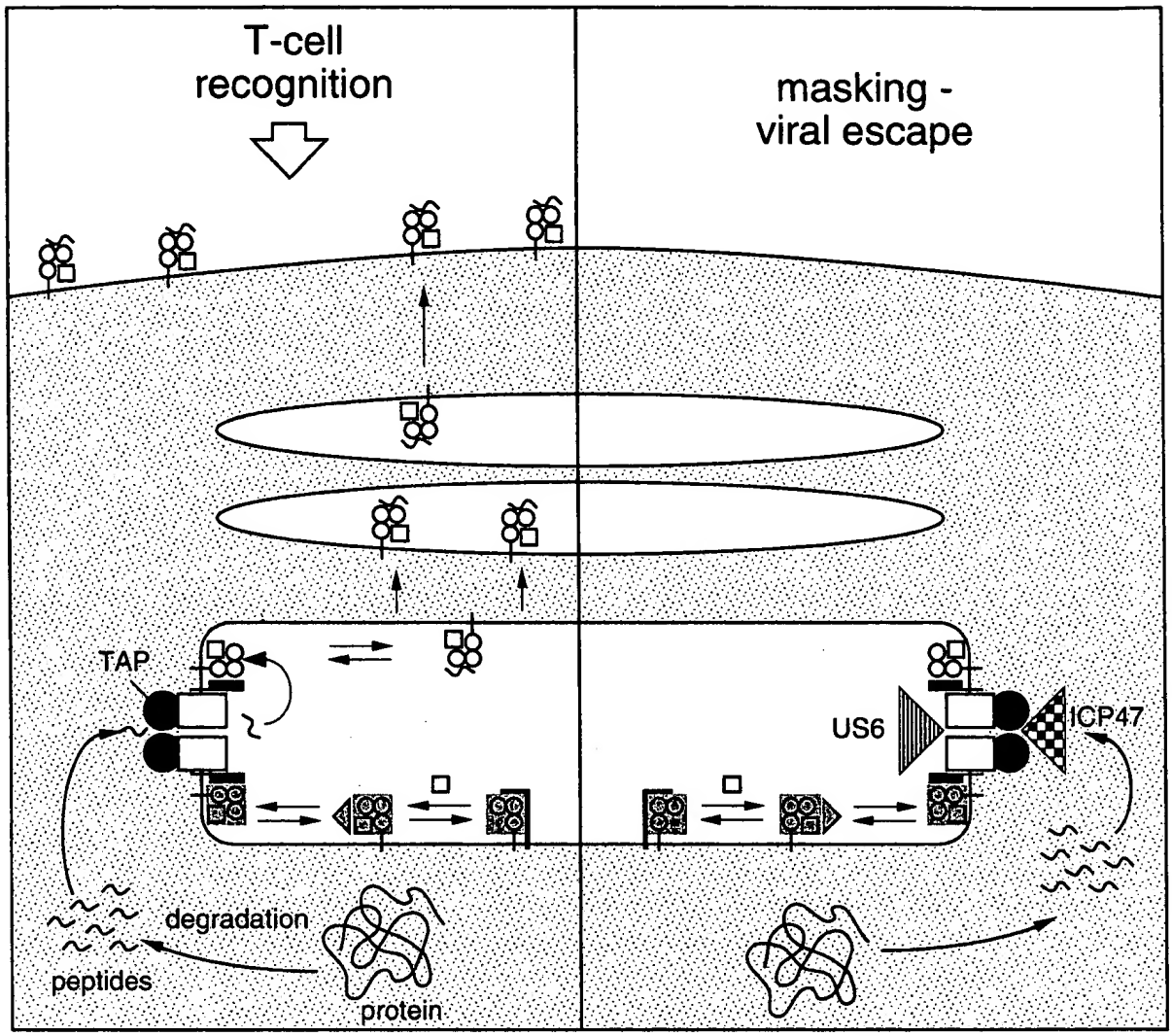

Fig. 6. Viral escape mechanism of herpes simplex virus protein ICP47 and human cytomegalovirus protein US6 blocking TAP function from different compartments and by different mechanisms. The symbols are explained in Fig. 1.

different stages after infection (for review see [125]). In contrast to ICP47, the ER-resident transmembrane class I glycoprotein US6, expressed late post infection, binds probably to the ER-luminal part of TAP, thereby inhibiting peptide translocation [126–128]. US6 does not affect the association of tapasin, calreticulin and MHC class I molecules to TAP [126]. Moreover, the action of US6 can be overcome by TAP induction with INF-γ. In contrast to ICP47, which competes with peptides for the binding site on TAP, US6 inhibits neither peptide binding nor ATP binding to TAP (Fig. 6). Thus, it seems that US6 blocks the translocation step by interacting with the luminal part of TAP and prohibiting a conformational change required for peptide translocation.

Only little is known about congenital human TAP deficiency. Inherited TAP deficiency in a family caused by a stop codon at position 253 of TAP2 resulting in non-functional TAP has been described [129]. The homozygous TAP2−/− siblings in this family show a 100-fold decreased expression of MHC class I molecules on the cell surface. Cytotoxic

T lymphocytes were present in a reduced amount. Despite this TAP deficiency, these siblings do not show increased susceptibility to viral infection. In further studies with MHC class I molecules expressed in lymphoblastoid cell lines and cytotoxic T cells derived from these individuals it was observed that the antigens seem to be translocated to the ER lumen in a TAP-independent manner [130].

At present, the participation of TAP in autoimmune diseases is a matter of controversy. Whereas some publications report that TAP is linked to autoimmune diseases, others report the opposite. Thus, further studies will be necessary to elucidate the linkage of TAP to autoimmune diseases.

In tumor cell lines and tumor tissues, a loss of antigen presentation by MHC class I molecules is often observed and the downregulation of MHC class I surface expression can have various reasons (for review see [131,132]). In some malignant tumors, the deficient presentation of endogenous antigens on the cell surface is caused by a drastically reduced level of mRNA of TAP1, TAP2, LMP1 and LMP2 that could be restored by IFN-γ stimulation. In these renal cell carcinomas, the transcription of TAP and LMP seems to be downregulated by an unknown mechanism [133]. Another interesting example of a tumor escape mechanism was found in small lung cancer where a TAP1 (R659Q) mutant was identified [90]. Expression of MHC class I molecules on the cell surface could be restored by expression of wild-type TAP1 or adding exogenous peptides. R659 is localized between the C-loop and the Walker B motif, indicating that this mutation affects ATP binding or hydrolysis. The suppression of MHC class I molecules at the cell surface is a tumor escape mechanism from immune recognition for tumor cells. However, it should kept in mind that these mechanisms are more complex as cells lacking MHC class I molecules on the cell surface might become targets for natural killer cells.

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Immunization against tumor and minor histocompatibility antigens by eluted cellular peptides loaded on antigen processing defective cells*

Material eluted from RMA lymphoma or B6 spleen cells under acid conditions was fractionated by reverse phase high-performance liquid chromatography, and tested for ability to restore the sensitivity to cytotoxic T lymphocytes of the processing/presentation defective mutant line RMA-S. This allowed identification of three fractions (termed M1, M2 and M3) carrying B6 antigens recognized by cytotoxic T lymphocytes (CTL) elicited across the minor histocompatibility barrier A.BY anti-B6 (both H-2^b) and one fraction (termed T1) carrying a tumor antigen recognized by B6 anti-RMA CTL. By parallel runs of material from cell lysates over major histocompatibility complex class I affinity columns, the M2 and M3 antigens were defined as K^b restricted, and M1 and T1 as D^b restricted. Isolated fractions loaded onto RMA-S cells could be used to prime anti-minor histocompatibility antigen and tumor CTL *in vivo*. They could also be used for *in vitro* restimulation of spleen cells from mice that had been primed either by antigen-loaded RMA-S, or by wild-type RMA tumor cells and B6 splenocytes. The CTL generated by these methods were specific for the loading antigen, and they also recognized the antigen on the "physiological" target, *i.e.* RMA or B6 lymphoblasts. This system based on RMA-S as an immunization and target antigen reporter cell may be used for dissection of complex CTL responses, *e.g.* in studies of clonal composition and epitope dominance, or for studies of tumors that are poor stimulators of immunity.

1 Introduction

MHC class I molecules present intracellularly derived peptides for self-non-self discrimination by $CD8^+$ T cells. It was initially demonstrated that CTL can recognize short linear epitopes of cytoplasmic and nuclear proteins [1], and that synthetic peptides could mimic antigen epitopes recognized by CTL [2, 3]. Extra electron densities revealed by crystallographic data on MHC class I molecules were consistent with peptide binding in the polymorphic cleft [4]. More recently it has become possible to analyze naturally processed peptides directly. This is based on elution of antigenic material under acid conditions, either from complete cellular extracts, or from purified MHC class I molecules, followed by separation/purification of the material utilizing reverse phase high performance liquid chromatography (HPLC) [5–12]. Analysis with this approach have revealed a number of important features of MHC class I presented peptides: in particular, it appears that each allelic form of the MHC class I molecules imposes restrictions in terms of peptide length (octa- or nonamers) and certain preferred sequence motifs [12].

It has been possible to elute naturally processed peptides in responses to viral [6–7], tumor [13], minor histocompatibility (MiHa) [8–11] and major histocompatibility [11] antigens. In the latter case, the mutant lymphoma line RMA-S [14, 15] was utilized in the analysis based on loading of naturally processed antigens. This mutant line has a defect resulting in impaired peptide loading in the MHC class I pathway [16–18]. The phenotype of RMA-S is due to defective TAP2 genes [19, 20]; its single copy message contains a premature stop codon [21]. The TAP2 product is homologous to the transport proteins in the ABC (ATP-binding cassette) family, and is thought to mediate transport from the cytosol to the endoplasmic reticulum of peptides or a cofactor essential for peptide loading into the MHC class I molecules [19–31]. The defect in peptide loading is not absolute and at least some antigens can be presented, albeit with reduced efficiency [11, 32, 33]. RMA-S expresses reduced levels of MHC class I molecules, most of which behave as if they were devoid of antigen: they do not assemble efficiently, they do not present internally derived antigen but present the corresponding epitopes when added as external peptide [16, 18], they are unstable at 37 °C but can be stabilized at reduced temperature or by the addition of MHC class I binding peptides [17]. By manipulation of culture conditions, RMA-S cells can thus be induced to express high levels of class I molecules loaded homogeneously with one dominant species of peptide.

Our initial interest in RMA-S for CTL studies arose when it was found to escape the rejection response over a minor histocompatibility barrier as well as tumor-specific, re-

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Abbreviation: **MiHa:** Minor histocompatibility antigen

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stricted CTL, in spite of detectable levels of MHC class I at the cell surface as measured by FACS analysis and recognition by allo H-2^b-specific cytotoxic T cells [18]. The purposes of the present study was (i) to confirm that the resistance of RMA-S to CTL recognizing MiHa and tumor antigen was due to lack of naturally processed peptide (as already shown for viral and allospecific CTL epitopes [11, 19]) and if so (ii) to test whether the RMA/RMA-S model could be used for dissection of epitopes in responses to MiHa and tumor antigens, and (iii) if homogenous loading of RMA-S cells with naturally processed antigen could be used to stimulate CTL *in vivo* and *in vitro* to yield responses with a limited epitope specificity. In the last approach, one long-term goal would be to develop a method for efficient, high antigen density immunization of T cell responses where the sequence of the relevant epitope(s) is unknown.

2 Materials and methods

2.1 Mice

The inbred strains C57BL/6 (H-2^b) and A.BY (H-2^b) were bred and maintained at the Department of Tumor Biology, Karolinska Institutet.

2.2 Cell lines

RMA and RMA-S are both derived from the Rauscher leukemia virus-induced mouse T cell lymphoma RBL-5 of C57BL/6 origin (H-2^b) [14]. T2D^b and T2K^b are D^b and K^b transfectants of the mutant human subline T2 (.174 × CEM.T2), which originates from T1 (.174 × CEM.T1) [34]. The cells were grown in RPMI 1640 medium supplemented with penicillin-streptomycin, 3×10^{-5} M 2-ME, and 5% FCS in 50 ml cell culture flasks at 37 °C and 5% CO_2.

2.3 Acid elution of naturally processed peptides from intact cells

RMA cell and B6 splenocytes (1×10^9–5×10^9, respectively) were washed in PBS × 3 and then added 10 ml PBS containing trifluoroacetic acid (TFA) to obtain pH 2–3 (usually 0.6–0.7% TFA). The preparations were centrifuged in aliquots and the supernatants recovered within 1 min after addition of TFA in PBS. The supernatants were filtered sequentially through MICROSEP™ MICRO-CONCENTRATORS (FILTRON, Filtron Technology Corporation, Northborough, MA) with cut-off limits of 30 and 10 kDa. The filtered material was either dried by vacuum centrifugation or separated on HPLC. All work was performed at 4 °C.

2.4 Acid elution of naturally processed peptides from immunoaffinity column-isolated MHC class I molecules

RMA cells and B6 splenocytes (5×10^9–10×10^9 respectively) were washed in PBS × 3 and lysed by stirring the cells for 60 min in 20 ml PBS containing 0.5% NP40. The lysates were centrifuged at 14 000 rpm for 30 min using a Sorvall RC-5C centrifuge and a SS34 rotor (Sorvall Instruments, Du Pont, Instrument AB Lambda, Stockholm). The pH of the supernatants were adjusted to 8.0 by adding 1 M Tris-HCl buffer pH 8.0. The lysates were pre-cleared by running through columns with Pharmacia 4CL-protein A-Sepharose beads loaded with antibodies from normal mouse serum. In a second step the pre-cleared lysates were run through immunoaffinity columns specific for different MHC class I products [Pharmacia 4Cl-protein A-Sepharose beads loaded with either AF6-88.5.3, (K^b α1α2-specific mAb) or 28-14-8S, (D^b α1α2-specific mAb)]. Binding of antibodies to protein A-Sepharose beads was performed as previously described [35]. Prior to elution of the bound MHC class I molecules, the columns were washed with 10 mM Tris–HCl buffer pH 8.0. Loaded beads were subject to acid extraction using 100 mM glycin pH 3.0. Bed volumes, 2 ml; flow rates, 0.5 ml/min. TFA was added to the collected material to a final concentration of 0.1%, which then was incubated during 15 min or swirling. All work was performed at 4 °C. The collected material was either dried by vacuum centrifugation or separated on HPLC.

2.5 Separation by reverse phase HPLC

The separations were performed on a reverse phase Superpac Pep S column (C2/C18, 5-μm particles, 4.0 × 250 mm; Pharmacia LKB, Uppsala) using Pharmacia LKB equipment. Elution procedure: solution A, 0.1% TFA in H_2O; solution B, 0.1% TFA in acetonitrile; 0–5 min, 100% A; 5–45 min, linear increase to 60% B; 45–50 min, 60% B; 50–55 min, linear decrease to 0% B. Flow rate 1 ml/min. Elution was monitored by measuring UV-light absorption at 214 nm in a continuous flow detector, and 1 ml fractions were collected. Individual fractions were dried by vacuum centrifugation.

2.6 *In vivo* priming

The mice were pre-immunized with three s.c. injections of either B6 spleen cells (10^7), RMA (2×10^6), RMA-S (2×10^6), RMA-S + peptide (2×10^6 RMA-S cells incubated with 100 μl of one HPLC fraction in a total volume of 1 ml for 12 h at 26 °C), or peptide alone (100 μl of one HPLC fraction). The preparations were irradiated with 10 000 rad prior to inoculation.

2.7 *In vitro* restimulation

Single-cell suspensions from spleens of pre-immunized and control mice were prepared. Effector cells 20×10^6 were incubated with preparations identical to those used for the *in vivo* primings. The cells were kept in 15 ml of RPMI 1640 medium supplemented with penicillin-streptomycin, 10% FCS and 3×10^{-5} M 2-ME. Cultures were kept at 37 °C and 5% CO_2 for 5 days.

2.8 *In vitro* cytotoxicity assay

Target cells were labeled with ^{51}Cr according to standard procedures [36] and resuspended in cell culture medium.

Peptide material (vacuum-dried HPLC fractions, or 200 μg of the synthetic influenza peptide 1968 NP 366–374) was disolved in 200 μl of PBS and aliquots of 10 μl were plated out on 96-well round-bottum microtiter plates. Target cells (5×10^3) were added to each well followed by addition of effector cells. Assay plates were incubated for 4 h at 37 °C and then harvested. Radioactivity was measured in a Pharmacia-LKB γ-counter (Pharmacia LKB) and specific lysis was calculated (cpm released with effector cells-cpm released without effector cells)/(cpm released by detergent-cpm released without effector cells) × 100.

3 Results

3.1 Analysis of CTL target antigens eluted under acid conditions from intact cells

RMA lymphoma and B6 spleen cells were incubated in PBS containing TFA (pH 2–3) for 1 min. The cell membrane of the cells appeared to remain intact when the treatment was kept this short, since the cells showed no increase of spontaneous ^{51}Cr release or of uptake of trypan blue (data not shown). The filtered supernatant was then either separated on a reverse phase HPLC column or directly dried in a vacuum centrifuge. The total material and the isolated fractions from the HPLC runs were tested for ability to sensitize ^{51}Cr-labeled RMA-S targets for recognition by MiHa-immunized (A.BY anti-B6) or tumor-immunized (B6 anti-RMA) CTL. Without loading of eluted material, RMA-S was completely resistant, while RMA was efficiently killed (Fig. 1). Incubation of RMA-S with unfractionated material from acid elution rendered it sensitive to killing by both effectors (data not shown). Reverse phase HPLC resolved the anti-minor alloantigen response in three main peaks, hereafter termed M1, M2 and M3 (Fig. 1A and B). One HPLC fraction of the material derived from RMA cells contained antigen detected by the tumor-specific CTL, hereafter termed T1 (Fig. 1C). None of the fractions of the B6 splenocyte-derived material could sensitize for tumor-specific CTL recognition (Fig. 1D). The procedure was highly reproducible for independent elutions and HPLC runs, with two reservations: (i) antigen activity usually appeared within one or two isolated 1-ml fractions, although one extra neighboring fraction sometimes was positive. (ii) M3 was not detectable in all experiments.

3.2 Analysis of CTL target antigens eluted under acid conditions from immunoaffinity-isolated MHC class I molecules

Cell lysates of RMA tumor cells and B6 splenocytes respectively were passed over immunoaffinity columns loaded with either antibodies from normal mouse serum, K^b-, or D^b-specific antibodies. After incubation with 0.1% TFA, the eluates were separated by reverse phase HPLC. This procedure generated one fraction from the anti-D^b column and two fractions from the anti-K^b column that sensitized RMA-S targets for minor alloantigen-specific CTL (Fig. 2A–D). The position of the fractions corresponded well to those isolated after elution under acid conditions without detergent lysis. We therefore tentatively assigned M2 and M3 as K^b-restricted and M1 as D^b-restricted, even if it remains to be formally proven that the material obtained by acid elution from intact cells and from immunoaffinity-purified MHC class I molecules represent identical epitopes. The tumor-specific CTL identified one fraction of the RMA-derived material isolated from the

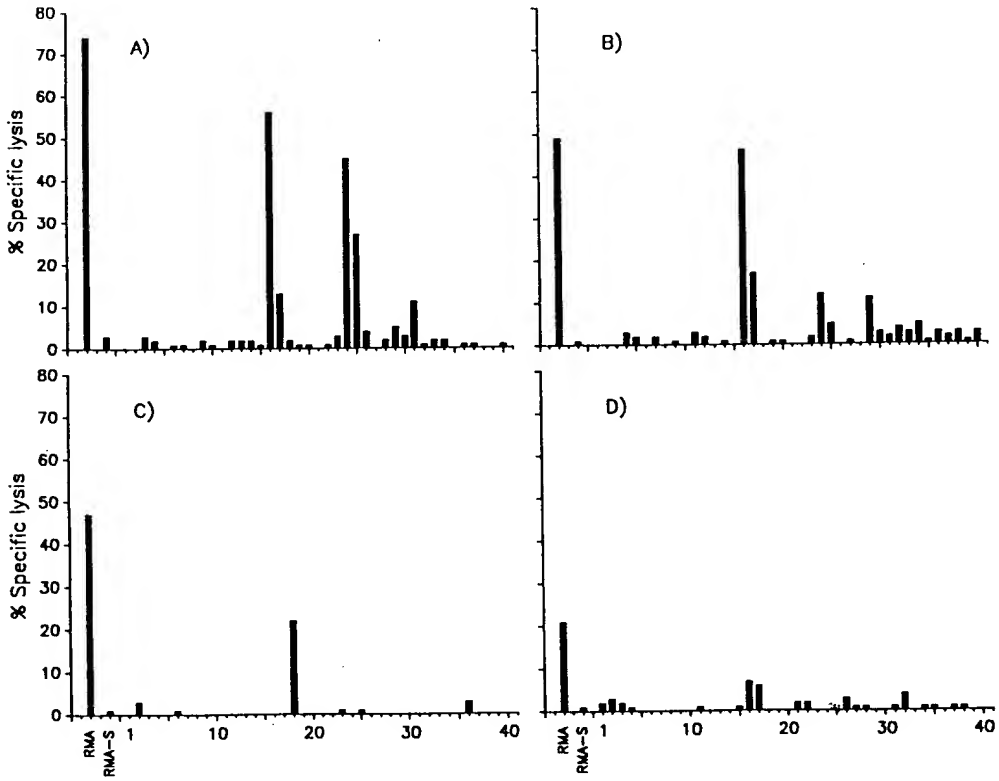

Figure 1. Functional identification of MiHa and a tumor antigen after elution of peptides under acid conditions from intact cells. Effector cells: MiHa-specific CTL (A.BYanti-B6) (A, B), and tumor antigen specific CTL (B6 anti-RMA) (C, D). Target cells: RMA (first bar A–D), RMA-S (second bar A–D) and RMA-S + aliquots of HPLC fractions (no. 6–47) derived from RMA eluates (A, C) and B6 splenocyte eluates (B, D).

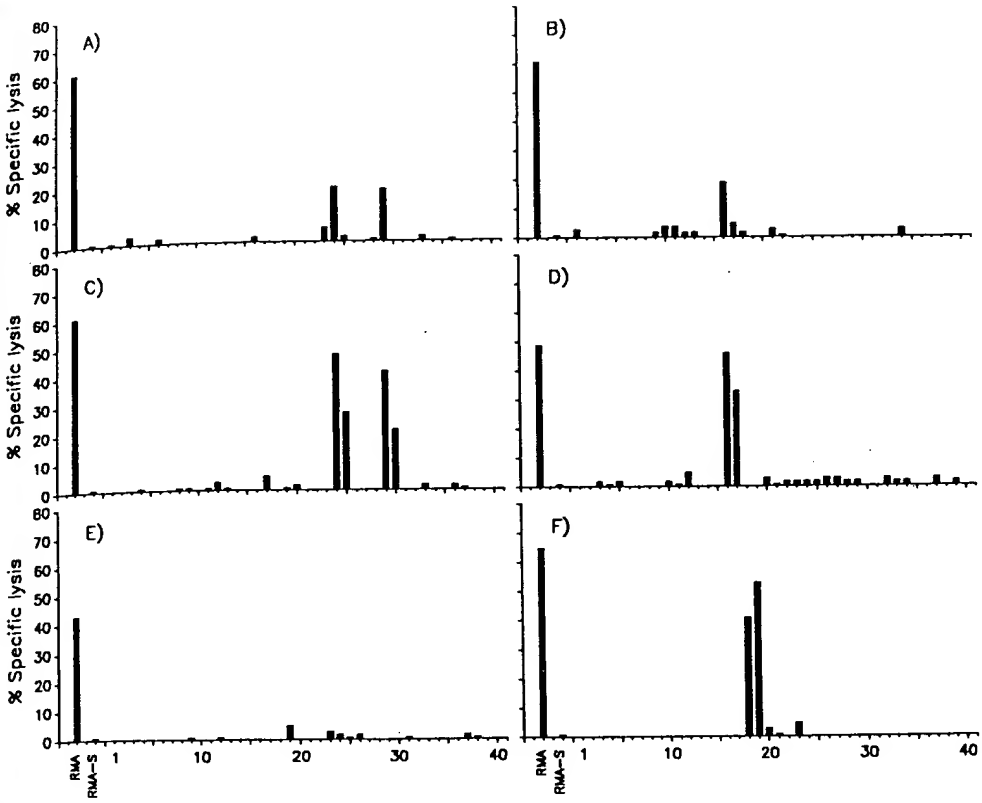

Figure 2. MHC class I allele restriction of three minor allo-antigens and one tumor antigen. Effector cells: MiHa-specific CTL (A.BY anti-B6) (A–D), and tumor antigen-specific CTL (B6 anti-RMA) (E, F). Target cells: RMA (first bar A–F), RMA-S (second bar A–F), and RMA-S + aliquots of HPLC fractions (no. 6–47) derived from K^b eluates from B6 splenocytes (A), RMA (C, E) and D^b eluates from B6 splenocytes (B), RMA (D, F).

anti-D^b column corresponding to the position of the previously defined fraction T1 (Fig. 2F), whereas no HPLC fraction of the K^b-derived material could sensitize for lysis by the same CTL (Fig. 2E). The restriction element for each antigen was confirmed in experiments where human T2 cells transfected with H-$2K^b$ or D^b were sensitized with the different HPLC fractions of eluates from intact cells and then used as targets for CTL. A.BY anti-B6 CTL killed T-$2K^b$ loaded with M2 and M3, and T-$2D^b$ loaded with M1 (Fig. 3A). Tumor antigen-specific CTL (B6 anti-RMA) recognized $T2D^b$ loaded with T1 (Fig. 3B). Reciprocal combinations (M2 and M3 on $T2D^b$, M1 and T1 on $T2K^b$) did not induce killing above background (data not shown). None of the identified antigens could be assigned to any of the major peaks of the reverse phase HPLC elution profiles (Fig. 4A and B).

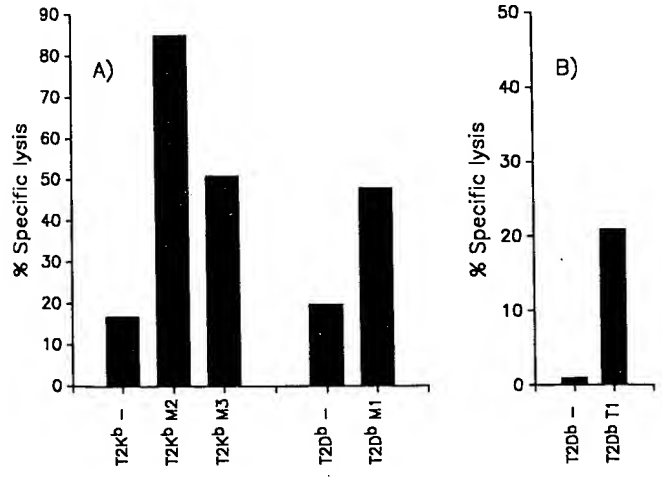

Figure 3. MHC class I restriction pattern of the minor allo-antigens and the tumor antigens eluted from intact cells under acid conditions. Effector cells: MiHa-specific CTL (A.BY anti-B6) (A), and tumor antigen-specific CTL (B6 anti-RMA) (B). Target cells: $T2K^b$ ($T2K^b$ –) (A), $T2K^b$ loaded with MiHa M2 ($T2K^b$ M2) (A) and MiHa M3 ($T2K^b$ M3) (A), $T2D^b$ ($T2D^b$ –) (A, B), $T2D^b$ loaded with MiHa M1 ($T2D^b$ M1) (A) and tumor antigen T1 ($T2D^b$ T1) (B).

3.3 Restimulation of CTL *in vitro* by RMA-S cells loaded with naturally processed antigen

As a first step to test whether the isolated material could also be used to stimulate CTL in the afferent arm of the response, we used RMA-S cells loaded with material from HPLC fractions of naturally processed antigen for restimulations *in vitro*. Responder cells were prepared from spleens from either A.BY mice immunized with B6 spleen cells (MiHa priming) or from B6 mice immunized with RMA cells (tumor antigen priming). As seen in Fig. 5, RMA-S loaded with M1 (RMA-S M1) stimulated B6-primed A.BY spleen cells to develop a cytotoxic response effective against RMA-S M1. These CTL did not kill RMA-S T1 (Fig. 5), RMA-S M2, RMA-S M3, RMA-S NP 366–374 or RMA-S without loaded antigen (data not shown). The killing was thus specific for targets expressing the antigen used to load the restimulating cells. RMA-S loaded with T1 induced B6 CTL that were specific for RMA-S cells loaded with the same antigen (Fig. 5). Note that RMA, expressing the antigens "physiologically", were killed by the CTL restimulated with M1 or T1-loaded RMA-S cells (Fig. 5).

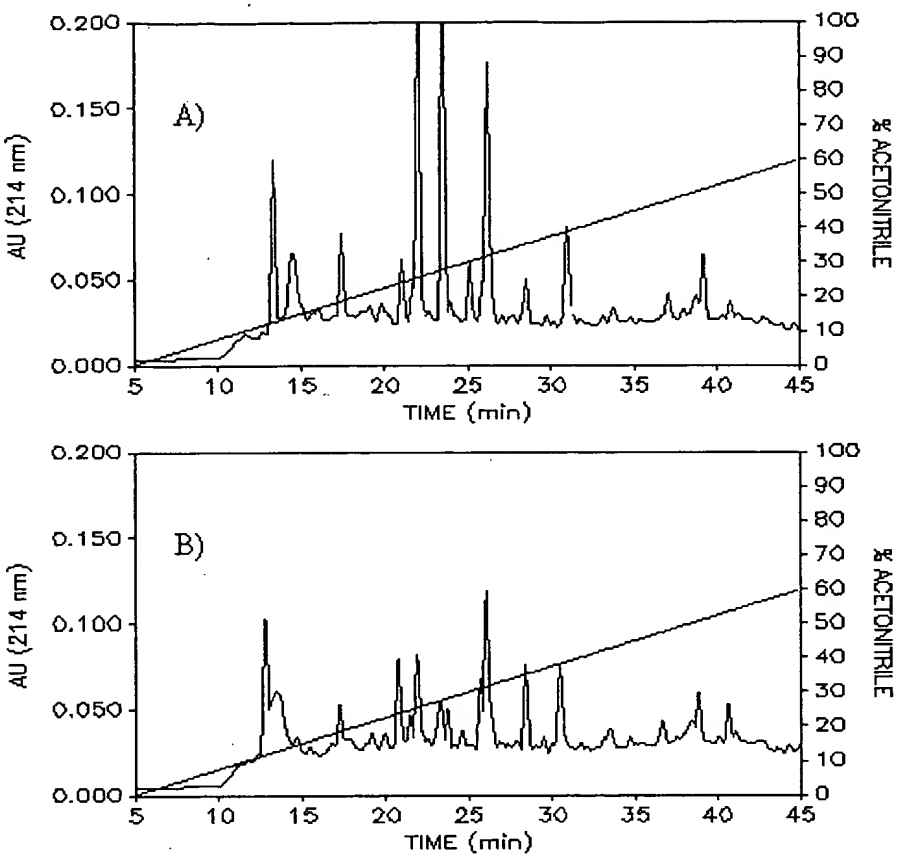

Figure 4. Reverse phase HPLC elution profiles of RMA tumor cell-derived antigens. Material eluted from immunoaffinity column purified K^b- (A) and D^b-molecules (B).

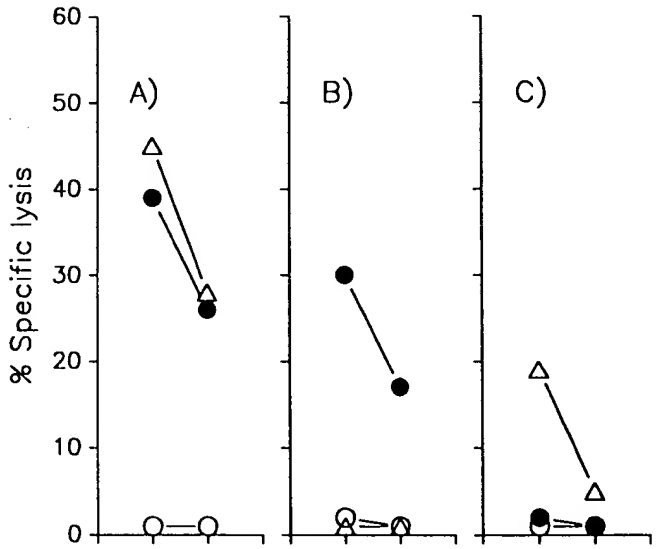

Figure 5. Generation of peptide-specific cytotoxic T cells from mice primed with intact spleen or tumor cells *in vivo* and restimulated *in vitro* with naturally processed and eluted antigens. Effector cells: A.BY primed in *vivo* against B6 and *in vitro* restimulated with either RMA-S (O–O) (A, B), RMA-S loaded with MiHa M1 (●–●) (A, B), and RMA-S loaded with tumor antigen T1 (△–△) (A, B). B6 primed *in vivo* against RMA and restimulated with RMA-S (O–O) (C), RMA-S loaded with MiHa M1 (●–●) (C) or RMA-S loaded with tumor antigen T1 (△–△) (C). Target cells: RMA (A), RMA-S loaded with MiHa M1 (B), and RMA-S loaded with tumor antigen T1 (C). Effector : target ratios 9:1 and 3:1.

3.4 *In vivo* priming of CTL responses by RMA-S cells loaded with naturally processed antigen

We then tested whether naturally processed antigen loaded onto RMA-S could be used throughout the afferent arm of the response, including the *in vivo* priming step. RMA-S M1 primed and restimulated A.BY CTL that could efficiently kill RMA and RMA-S M1 (Fig. 6, Table 1). Similarly, RMA-S T1 primed and restimulated B6 CTL that could kill the syngeneic wild type tumor RMA (Table 2). We have so far been unable to obtain primary *in vitro* stimulation of naive spleen cells by co-culture with RMA-S loaded with naturally processed antigen (Table 1). Note also that it was necessary to load RMA-S with eluted antigen in the *in vivo* priming step as well as in the *in vitro* restimulation step to induce CTL.

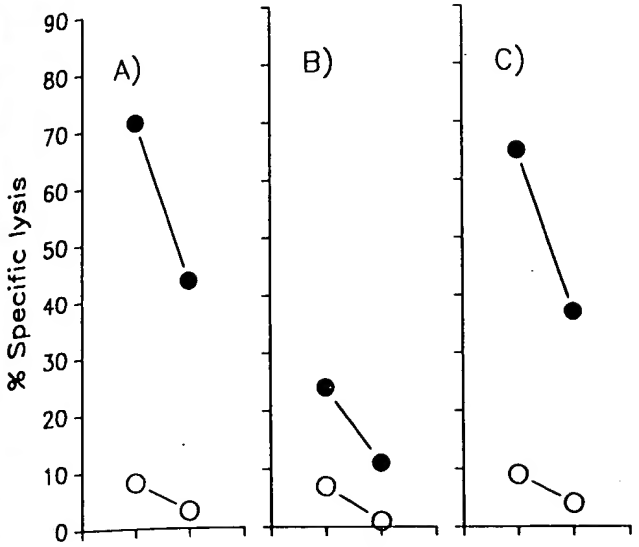

Figure 6. Generation of peptide specific cytotoxic T cells by *in vivo* priming and *in vitro* restimulation with a naturally processed and eluted MiHa. Effector cells: A.BY splenocytes primed *in vivo* and restimulated *in vitro* with RMA-S (○–○) (A–C), and RMA-S loaded with MiHa M1 (●–●) (A–C). Target cells: RMA (A), RMA-S (B) and RMA-S loaded with MiHa M1 (C). Effector : target ratios 9 : 1 and 3 : 1.

Table 1. Generation of minor histocompatibility antigen-specific CTL[a]

A.BY effector cells		Target cells		
In vivo immunization	*In vitro* restimulation	RMA-S	RMA-S M1	RMA-S NP
RMA-S	RMA-S	6, 5, 2[b]	10, 5, 6	9, 4, 1
–	RMA-S M1	0, 0, 0	2, 5, 2	1, 1, 0
RMA-S M1	–	1, 0, 0	2, 1, 3	2, 1, 0
RMA-S M1	RMA-S M1	21, 9, 4	54, 50, 37	17, 9, 4

a) *In vivo* and *in vitro* stimulation of antigen-specific cytotoxic T cells with a naturally processed and eluted minor alloantigen. Effector cells: A.BY splenocytes unprimed (–), primed *in vivo* with RMA-S, or primed *in vivo* with RMA-S loaded with M1 (RMA-S M1), were stimulated *in vitro* with RMA-S, or RMA-S M1, or cultured alone. Target cells: RMA-S, RMA-S M1 and RMA-S loaded with influenza (1968) nucleoprotein peptide 366–374 (RMA-S NP).

b) % Specific lysis at effector : target ratios 15, 5, 1 : 1.

Table 2. Generation of tumor antigen-specific CTL[a]

C57BL/6 effector cells		Target cells	
In vivo immunization	*In vitro* restimulation	RMA	RMA-S
RMA-S	RMA-S	11, 6, 2[b]	2, 3, 0
T1	T1	0, 0, 0	0, 0, 0
RMA-S T1	T1	1, 0, 0	4, 0, 0
T1	RMA-S T1	0, 1, 0	0, 0, 0
RMA-S T1	RMA-S T1	43, 31, 16	7, 5, 0

a) *In vivo* and *in vitro* stimulation of antigen-specific cytotoxic T cells with a naturally processed and eluted tumor antigen. Effector cells: B6 splenocytes primed *in vivo* with RMA-S, RMA-S loaded with tumor antigen T1 (RMA-S T1), or tumor antigen T1 alone (T1). Target cells: RMA and RMA-S.

b) % Specific lysis at effector : target ratios 15, 5, 1 : 1.

4 Discussion

The sensitivity of antigen presentation (TAP2)-defective RMA-S cells to bulk CTL, specific for minor alloantigen and tumor antigen, was restored by incubation of eluted cellular peptides from B6 splenocytes or RMA tumor cells. Application of techniques for analysis of MHC class I-bound cellular peptides [5–13] led to identification of four fractions containing MiHa termed M1 (restricted by H-2D^b), M2 and M3 (both restricted by H-2K^b) and one tumor antigen termed T1 (restricted by H-2D^b). We refer to the former as MiHa since they consistently dominated the A.BY anti-B6 CTL response; sensitivity to this CTL response is required for rejection of RMA across this minor histocompatibility barrier [14]. The role of each of these antigens in for example skin graft rejection remains to be determined. T1 is referred to as a tumor antigen since the CTL recognizing this antigen were generated by stimulation of syngeneic B6 spleen cell effectors with RMA tumor cells, and the antigen was not detected in peptide eluates from B6 spleen cells. The observed D^b restriction of T1 is in line with earlier studies showing that the common antigen in tumors induced by Friend, Moloney, and Rauscher (FMR) C-type viruses is recognized in the context of H-2D^b when both effector and target cells express this MHC class I allele [37, 38]. Previous biochemical characterisations of the common FMR antigen identified a glycoprotein of 175 kDa plus a protein of 50 kDa [39] and excluded the viral proteins as the source of antigen [40]. In light of the novel peptide presentation paradigm, these interpretations should be considered with caution and the identity of the antigen remains an open question.

The HPLC separations performed did not result in base line resolution of the individual eluted peptides (Fig. 4A and B) and the collected fractions are expected to contain several peptide species. Further analysis by capillary electrophoresis indeed indicate that fractions with antigen activity (for example M1 and T1) contain at least half a dozen peptide species (data not shown). This is well in line with reports on mass spectrometry analyses of HPLC fractionated peptide eluates [41]. Preparative capillary electrophoresis is currently being applied to achieve further resolution with the goal of isolating single peptide species for functional testing and sequencing.

So far, no minor histocompatibility or tumor antigen published has been identified by sequencing of eluted cellular peptides. Van der Bruggen et al. have however eluted a peptide which co-purified with a synthetic peptide previously identified (by gene cloning, sequencing and synthetic peptide screening) as tumor antigen in human melanoma [42]. Certain abundant peptides from eluates of isolated MHC class I molecules have been sequenced after isolation [5–7, 43, 44]. Most CTL epitopes are however not present in amounts sufficient to yield major peaks in HPLC profiles allowing purification in one step [44].

It was possible to use RMA-S cells loaded with HPLC fractions of eluted cellular material to prime *in vivo* and restimulate *in vitro* for determinant specific CTL responses. Various procedures have previously been applied to immunize for CTL responses with synthetic peptides, available in larger quantities [45–51]. The novel aspect of this work is that the use of peptide-loaded TAP2-deficient

cells allowed immunization with limited amounts of naturally processed peptides eluted from cells. The procedure should be applicable to dissect the afferent as well as efferent arm of CTL responses to challenges involving several class I-presented determinants, even when the sequences of the epitopes have not been defined, *e.g.* for MiHa [52, 53].

The peptide loading and immunization procedure may also be applied to identify antigens in tumors which are poor stimulators (and often even inhibitors) of cell-mediated responses *in vitro* (polyoma virus and methylcholanthrene-induced tumors represent two such examples in the mouse). The problems with such cells may be possible to circumvent by using them as peptide donors to TAP-deficient cells such as RMA-S or MHC class I-transfected insect cells [54] and letting these act as antigen carrier and reporter cells. Priming with antigen-loaded cells *in vivo* may be necessary, since we have so far failed to induce primary CTL responses *in vitro* with eluted antigens loaded onto RMA-S cells (Tables 1 and 2). This has however been possible with synthetic peptide corresponding to the Sendai virus nucleoprotein 324–332 epitope [49]. The amount of loaded antigen may be critical; synthetic peptides were used in nanomole amounts, while we estimate from further biochemical analysis that the eluted antigen was applied in the femtomole range.

Using the approach described here, it may be possible to dissect and analyze epitope dominance in the afferent and efferent arms of responses to tumor, viral and minor histocompatibility antigens. The method can be applied to immunize specifically for CTL to certain epitopes, including those against which the response may be otherwise masked or interfered with by other antigens [52, 53]. This can be particularly important in responses to heterogeneic tumors and perhaps also autoantigens, which may be "cryptic" in many cases [55]. The procedure described here may be a useful tool in functional and structural analysis of tumor and minor histocompatibility antigens, as an alternative and complement to approaches based on molecular genetics [42].

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Derivation of HLA-A11/K^b Transgenic Mice

Functional CTL Repertoire and Recognition of Human A11-Restricted CTL Epitopes[1]

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Transgenic mice expressing chimeric human (α1 and α2 HLA-A11 domains) and murine (α3, transmembrane, and cytoplasmic H-2K^b domains) class I molecules were derived. These mice were used as a model system to study the immunogenicity of human CTL epitopes and also to examine the aspects of Ag processing differences of mice vs man. Immunization of these mice with seven known HLA-A11-restricted CTL epitopes emulsified in IFA resulted in vigorous specific CTL responses. A larger panel of 45 A11-binding peptides was used to examine the relationship between immunogenicity in the HLA-A11/K^b transgenic mice and HLA-A11 binding capacity. Twenty-one of 28 (75%) peptides with high binding affinities (50% inhibitory concentration (IC_{50}), 2–50 nM) and 7 of 13 (54%) intermediate binding peptides (IC_{50}, 50–500 nM range) were immunogenic. In parallel, 19 of these peptides were used for in vitro primary immunizations of PBMC derived from HLA-A11 healthy human donors. It was found that 8 of 8 peptides that were able to elicit CTL in primary human in vitro cultures were also immunogenic in HLA-A11/K^b mice. Finally, HLA-A11/K^b transgenic mice were found to generate an A11/K^b restricted CTL response following immunization with influenza virus A/PR/8/34, suggesting that, at least to some extent, A11 epitopes are generated by transgenic mice as a result of natural in vivo processing and presentation. *The Journal of Immunology,* **1997, 159: 4753–4761.**

Activation of cytotoxic T cells depends on engagement of their Ag-specific TCR by bimolecular complexes consisting of specific peptides presented in the context of class I molecules. This receptor/ligand interaction is an essential step in the generation of immune responses to virally infected or cancerous cells (for review, see Refs. 1–4).

Understanding of class I-restricted Ag processing and presentation has greatly expanded within the last few years. Proteasomes are the major proteolytic component responsible for generation of antigenic peptides by processing of protein Ags (for review, see Refs. 5–10). Once generated, peptides are transported by the heteromeric transporter complex, TAP1/TAP2, across the membrane of the endoplasmic reticulum where they can associate with newly synthesized class I heavy chain and β_2-microglobulin (β_2m)[3] (11–18). Aside from an apparent preference for peptides of 8 to 16 residues in length (15, 19–21), the predominant specificity of the TAP complex has been found to be associated with the COOH termini of the peptides. Human TAP molecules import hydrophobic and positively charged C termini. In contrast, the murine TAP prefers hydrophobic C termini and has been reported to have a poor capacity to transport peptides with positively charged C termini (22–24). Following peptide binding to class I molecules, peptide/class I MHC complexes are transported to the cell surface where they are available for recognition by Ag-specific T cells.

Interestingly, the different specificity of murine and human TAP molecules is also reflected in the peptide binding motifs of class I molecules of the corresponding species. Several class I molecules bind peptides with hydrophobic C termini, in both mice and humans (H-2K^d, -D^d, -L^d, -K^b, -D^b, -K^k in mice, and HLA-A2.1, -A24, -B7, -B8, in humans). By contrast, class I molecules that preferentially bind peptides with positive charges at their C termini have been identified in humans (e.g., HLA-A3, -A11, -B27) but not in mice (25).

An independent set of studies has investigated the use of HLA class I transgenic mice as a model system to study immunogenicity of CTL epitopes and immunodominance in CTL responses (26). To preserve species-specific interactions between CD8 and the α3 domain of class I molecules, a hybrid class I molecule, encompassing the leader, α1, and α2 domains of human HLA-A2.1 fused to the α3, transmembrane, and cytoplasmic domains of the murine class I molecule H-2K^b, was constructed. At the immunologic level, A2.1/K^b transgenic mice were validated by demonstrating that the CTL response elicited by infection with the influenza virus A/PR/8/34 was directed against the same dominant epitope recognized in humans expressing the HLA-A2.1 Ag. Subsequent studies (27) using the same HLA-A2.1/K^b transgenic mice and a panel of 38 different, previously defined, synthetic epitopes, expanded these observations and demonstrated that a good correlation existed between the CTL repertoire of these mice and HLA-A2.1-positive

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[3] Abbreviations used in this paper: β_2m, β_2-microglobulin; IC_{50}, 50% inhibitory concentration; HBV, hepatitis B virus; HCV, hepatitis C virus; PAP, prostatic acid phosphatase; MAGE, melanoma antigen; EBNA, Epstein-Barr virus-encoded nuclear antigen; MFC, mean channel fluorescence; c, core (e.g., HBVc); pol, polymerase.

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human individuals. Other studies demonstrated the utility of a HLA-A2.1 transgenic murine model in predicting the potency of CTL epitopes in humans (28–31).

Although the A2.1 Ag is found in a large portion of the human population (20–50%, depending on the particular ethnic group considered) (32, 33), development of epitope-based vaccines with broad population coverage requires validation and use of model systems to study CTL responses restricted by other common HLA types.

In the present study, we describe the development of HLA-A11/K^b transgenic mice. The HLA-A11 Ag is one of the most common class I HLA Ags, expressed in 4 to 33% of the general population, depending on the ethnic background considered (34). It binds peptides characterized by a somewhat hydrophobic residue in position 2 (A, L, I, V, M, S, or T) and positively charged residues (R or K) at the C termini (35–37). The preferred length appears to be about nine residues, although shorter and longer ligands have been reported (37, 38). Furthermore, the A11 Ag is also a member of a family of common HLA alleles that share a largely overlapping peptide binding motif. This family has been termed the A3-like supertype and includes, minimally, *A*0301*, *A*1101*, *A*3101*, *A33*, and *A*6801* (38, 39).

Materials and Methods

Peptides

Peptides were synthesized according to standard *t*-BOC or F-moc solid phase synthesis methods (40). Peptides were purified by HPLC and their identity verified by mass spectroscopy. Purity of peptides was usually >95% as determined by standard HPLC methods. Peptides were dissolved in DMSO at a concentration of 20 mg/ml, stored frozen at 20°C, and diluted in PBS before use.

Soluble HLA-A11 peptide binding assays

HLA peptide binding assays were performed as previously described (28, 36, 40). Briefly, purified HLA-A11 molecules, 1 μM of human β_2m (Scripps Laboratories, San Diego, CA), and 1 to 10 nM of radiolabeled A3CON1 (sequence KVFPYALINK; see Ref. 38) peptide were incubated in the presence of 0.05% Nonidet P-40/PBS (Fluka Biochemika, Ronkonkoma, NY) and a mixture of protease inhibitors (final concentration: 1 mM PMSF, 1.3 mM 1,10 phenanthroline, 73 μM pepstatin, 8 mM EDTA, and 200 μM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (all from Calbiochem, San Diego, CA)) for 2 days at room temperature. The percentage of labeled peptide bound to HLA-A11 was determined by gel filtration either alone or in the presence of a dosage range of each test peptide, so that the concentration of each test peptide necessary to inhibit 50% (IC_{50}) of the binding of the radiolabeled peptide could be determined. As a positive control for inhibition, in each experiment the A3CON1 was tested. The average IC_{50} for A3CON1 was 6 nM. The binding capacity of peptides was scored according to their IC_{50} as high (IC_{50}, < 50 nM), intermediate (IC_{50}, 50–500 nM), low (IC_{50}, 500–10,000 nM), and non-binders (IC_{50}, >10,000 nM).

Media, cell lines, and mice

Cells were expanded and maintained in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% FCS (Sigma Chemical Co., St. Louis, MO), 4 mM L-glutamine (Irvine Scientific, Santa Ana, CA), 5×10^{-5} M 2-ME (Sigma Chemical Co.), and 50 μg/ml gentamicin (Irvine Scientific). The cell line .221A11/K^b was derived by transfection of the HLA-A11/K^b gene into the .221 cell line, which lacks expression of HLA-A, -B, or -C class I genes due to γ-ray-induced deletions in the HLA complex (41). The A11/K^b gene was cloned into the RSV.5*neo* vector and electroporated (Bio-Rad Gene Pulser, Hercules, CA) into the .221 cell line, followed by selection of stable transfectants in 400 μg/ml of G418 (Life Technologies). These cells were expanded in the absence of G418 selection, but were periodically examined for expression of HLA-A11/K^b by comparative cytofluorometry. C57BL/6J mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Production of transgenic mice

Chimeric human-mouse class I cDNA constructs were used to generate transgenic mice and were produced by standard PCR amplification and cloning methods (42). These constructs contain the leader, α1, and α2 domains from HLA-A11 fused to the murine H-2K^b α3, transmembrane, and cytoplasmic domains. The sequence of all DNA constructs was confirmed by standard DNA sequencing methods. The class I hybrid construct was subsequently cloned into a cDNA expression vector consisting of components derived from a H-2L^d promoter, an intron, and a polyadenylation signal. To verify that the chimeric genes were appropriately expressed, the class I construct was transfected into CHO cells, stable populations selected, and surface expression of the class I protein molecule verified by FACS analysis. Subsequently, purified DNA was injected in fertilized BALB/c × C57BL/6 eggs for the production of transgenic mice, as previously described (43). The resulting progeny were screened for expression of the class I transgene by FACS using the HLA panreactive class I mAb, 9.12.1 (44). After backcrossing the highest expressing class I transgenic mice for four generations into C57BL/6 mice, homozygous transgenic mice were generated by heterozygous matings. Homozygosity was determined by backcrossing into C57BL/6 mice and was defined operationally as all offspring testing positive for transgene expression, with at least seven offspring tested (which gives a p value of 0.008 for parental homozygosity). Mice used in backcrossing experiments were purchased from The Jackson Laboratory.

Flow cytometry analysis of surface class I

Spleens from C57BL/6, HLA-A11/K^b, or HLA-A2.1/K^b transgenic mice were disrupted with a 15-ml Tissue Grinder (Kontes, Hayward, CA), and the resulting single-cell suspension was treated with NH_4Cl RBC lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2, EDTA, pH 7.2–7.4). Cell pellets resulting from individual spleens were resuspended in 1 ml of lysis buffer for 3 min, washed twice in RPMI 1640/10% FCS, and applied (up to 1.5×10^8 cells/column) to nylon wool columns (Cevis Corporation, San Diego, CA) for enrichment of T and B cells. Columns were incubated for 45 min at 37°C followed by collection of the flow-through (enriched T cells) in a volume of ~12 ml. To collect adherent cells (enriched B cells), the nylon wool was agitated by stirring with a 1-ml pipette followed by elution with 12 ml of 37°C medium. These semipurified T and B spleen cell populations were incubated with 100 μl of 9.12.1 (44) (human pan class I reactive), 28-14-8S (45) (anti-H-2D^b; American Type Culture Collection, Rockville, MD), mAb culture supernatants, or 100 μl of PBS/2% FCS in 96-well round-bottom plates (10^6 cells/well) (Linbro/Titertek ICN, Aukora, OH). After 30 min at 4°C, the cells were washed twice with 200 μl of PBS/2% FCS, and 100 μl of the secondary mAb (goat anti-mouse FITC-labeled Ab; Jackson ImmunoResearch, West Grove, PA), to a final concentration of 28 μg/ml, in PBS/2% FCS was added and further incubated for 30 min at 4°C. The cells were again washed twice with 200 μl of PBS/2% FCS and were then analyzed for MHC expression. Expression levels were determined by mean channel fluorescence (MFC) using a FACScan (Becton Dickinson Co., San Jose, CA).

CTL induction in A11/K^b transgenic mice induced by peptide immunization

HLA-A11/K^b mice were immunized by s.c. injection at the base of the tail with a mixture of HLA-A11-binding peptide (50 μg/mouse) and the helper IA^b-restricted epitope, hepatitis B virus core (HBVc) 128-140 (140 μg/mouse) (46) in PBS/10% DMSO emulsified in IFA. After 11 days, the mice were killed, and splenocytes (3×10^7 in 10 ml) in RPMI/10% FCS in T25 flasks (Costar, Cambridge, MA) were stimulated in vitro by the addition of 1 μg/ml of various peptides, in the presence or absence of syngeneic irradiated (3000 rad) LPS blasts (1×10^6 cells/ml) as an additional source of APC. After a 6-day incubation, cytotoxicity was measured using 10,000 ^{51}Cr-labeled A11/K^b-transfected .221 target cells, 1 μg/ml peptide, and varying numbers of effector cells in U-bottom 96-well plates (Costar). ^{51}Cr release was measured by removing 100 μl of supernatant after 4 h at 37°C; the percentage specific lysis was determined by the formula: percent lysis = 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. To facilitate comparison of responses from different experiments, data were also expressed in LU_{30} per 10^6 effector cells. One lytic unit (LU) is defined as the number of effector cells required to achieve 30% lysis of 1×10^4 ^{51}Cr-labeled target cells (30% lysis at E:T ratio of 100:1). Ten LU represents 30% lysis at the 10:1 ratio, 100 LU represents 30% lysis at the 1:1 ratio, and so on. CTL responses of ≥2 $LU_{30}/10^6$ cells were considered positive in analogy to previous studies (27, 28, 31, 47).

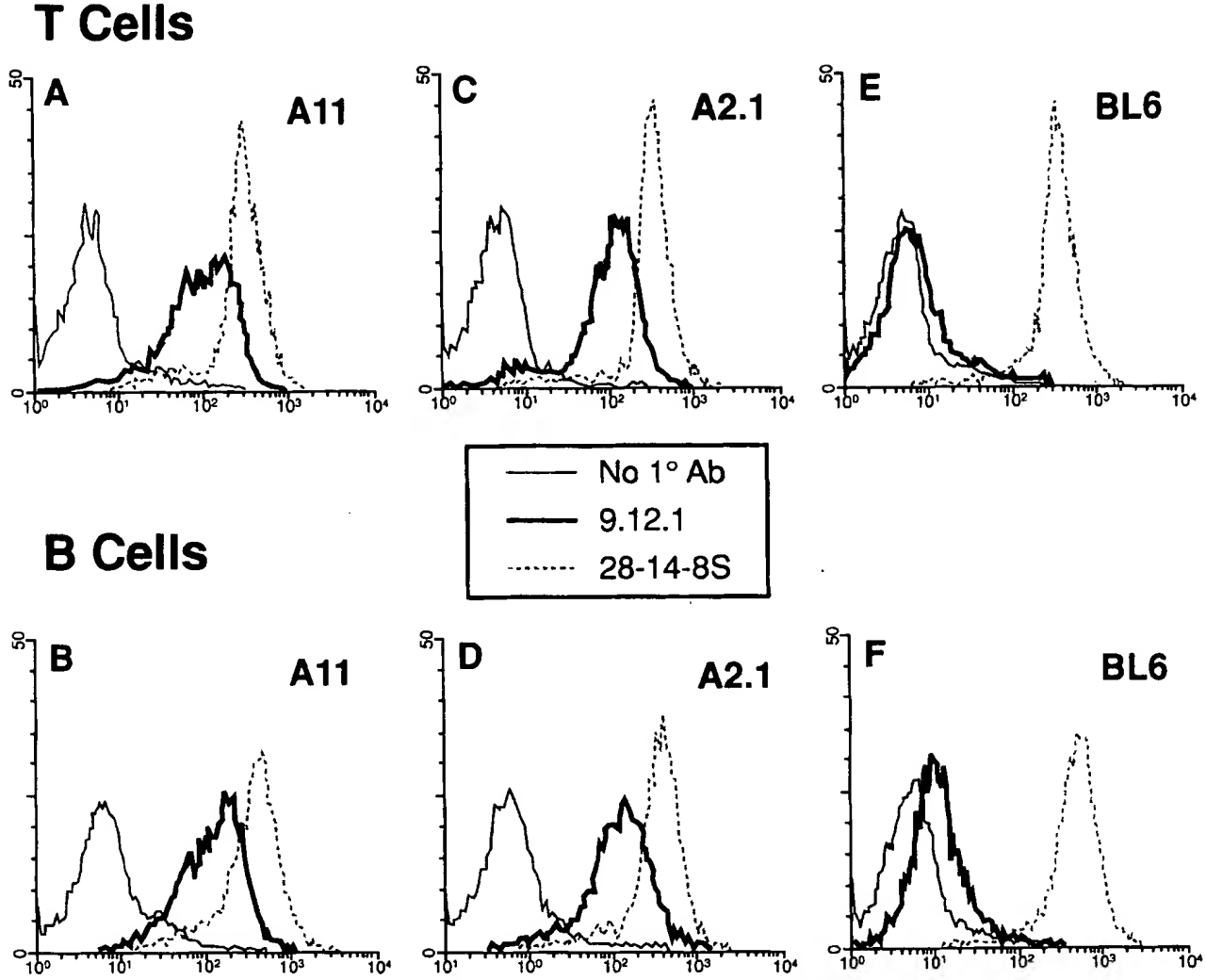

FIGURE 1. Comparison of cell surface expression of A11-K^b, A2.1/K^b, and endogenous H-2 class I on nylon wool-purified T and B splenocytes. HLA-A11/K^b (*A, B*), HLA-A2.1/K^b (*C, D*), and C57BL/6 (E, F) semipurified spleen cells were analyzed by cytofluorometry using human pan-class I-specific Ab 9.12.1 (44); H-2D^b-specific Ab 28-14-8S (45); or secondary goat anti-mouse FITC-labeled Ab only (no primary Ab). The FITC-labeled secondary Ab was specific for mouse IgG.

CTL in A11/K^b transgenic mice induced by influenza virus A/PR/8/34

Mice were primed i.p. with 100 to 300 hemagglutinating units of A/PR/8/34 influenza virus (SPAFAS, Storrs, CT). After 3 wk, spleens were removed and splenocytes pooled. In vitro secondary responses and CTL assays were performed as described above.

Induction of primary CTL responses

Induction of primary CTL in vitro responses from PBMC derived from normal donors was performed as described previously (48). Briefly, 2 × 10^6/ml of Ficoll-purified PBMC were cultured for 4 to 7 days in the presence of 0.005% fixed *Staphylococcus aureus* Cowan I strain (Pansorbin Calbiochem, La Jolla, CA), 20 μg/ml Immunobeads (rabbit anti-human IgM; Irvine Scientific), and 20 ng/ml human rIL-4 (Genzyme, Boston, MA). These stimulator cells were subsequently incubated for 2 min in citrate-phosphate buffer, pH 3.0, containing 1% BSA and 3 μg/ml β_2m. The buffer was neutralized with sodium phosphate buffer, pH 7.5, and the cells were centrifuged. The APCs were resuspended in PBS/1%BSA, 30 μg/ml DNase, 3 μg/ml β_2m, and 50 μg/ml test peptide, and then incubated for 4 h at 20°C. Subsequently, the cells were irradiated at 6100 rad, washed, and adjusted to 1 × 10^6 cells/ml.

Enriched $CD8^+$ effector T cells were prepared using AIS MicroCellector T-150 flasks (Applied Immune Sciences, Menlo Park, CA) specific for the depletion of $CD4^+$ T cells. $CD8^+$-enriched effector cells (3 × 10^6 cells/well) were plated with 1 × 10^6 cells/well of peptide-pulsed stimulator cells in a 24-well plate. A final concentration of 10 ng/ml rIL-7 (Genzyme) was added at the initiation of the culture and on day 7, and 10 U/ml rIL-2 (Sandoz, Basel, Switzerland) were added every 3 days thereafter. The cultures were restimulated twice and, following the last restimulation, the cultures were tested for cytolytic activity against peptide-pulsed, A11-expressing target cells (EBV-transformed B lymphoblastoid cell line, BVR) in a standard 5-h ^{51}Cr release assay, as described above.

Results

Production and characterization of HLA-A11/K^b transgenic mice

Homozygous A11/K^b transgenic mice were derived as described in the *Materials and Methods*. The pattern of A11/K^b expression of these mice is shown in Figure 1. The chimeric transgene is well expressed in both enriched T (Fig. 1*A*) and B cell populations (Fig. 1*B*), albeit at an approximately threefold lower level than the murine class I H-2D^b Ag (135–339 MFC vs 163–438 MFC in T and B cells, respectively). In fact, this level of HLA-A11/K^b expression is comparable with the expression level observed in the case of the previously characterized (26) HLA-A2.1/K^b transgenic mice (Fig. 1, *C* and *D*). This appears to be true both at the level of T

Table I. *Immunogenicity of known A11-restricted epitopes in A11/K^b transgenic mice*

Peptide	A11 Binding Capacity nM IC_{50}	CTL Response A11/K^b No. positive/No. tested	A11/K^b $LU_{30}/10^6$ cells[a]	C57BL/6J No. Positive/No. tested	C57BL/6J $LU_{30}/10^6$ cells
HBVc .141	4.0	6/6	12.1 (2.6)	0/3	—[b]
HIV pol .325	4.3	5/6	4.1 (1.7)	0/3	—
HIV nef .84	5.0	6/6	33.3 (1.4)	0/3	—
HBV pol .149	14.0	6/6	13.1 (1.2)	0/3	—
HBV pol .377	16.7	6/6	10.1 (2.5)	0/3	—
EBNA .416	18.0	5/6	28.7 (4.8)	0/3	—
HIV pol .507	20.6	5/6	6.2 (2.3)	0/3	—

[a] Geometric mean of positive cultures (x/÷ SD).
[b] — Indicates no response detected.

(Fig. 1*C*) and B (Fig. 1*D*) cell populations, which expressed the A2.1/K^b transgene at a level of approximately 135 and 158 MFC, respectively. Finally, by comparing class I H-2D^b expression levels in transgenic and C57BL/6 mice, it was noted that introduction of the A11/K^b transgene did not perturb or change the expression of the class I H-2 Ags to any significant extent (Fig. 1, *E* and *F*).

Immunogenicity of known dominant A11-restricted epitopes in HLA-A11/K^b transgenic mice

To assess the A11/K^b mice at the functional level, the immunogenicity in A11/K^b transgenics of seven known A11-restricted epitopes (HIV nef 84–94 (49); HIV pol 325–333 (50); HIV pol 507–518 (51); Epstein-Barr virus-encoded nuclear Ag (EBNA) 416–424 (52); HBVc 141–151 (53); HBV pol 149–159 (F. V. Chisari, unpublished data); and HBV pol 377–386 (F. V. Chisari, unpublished data)) was assessed. First, the binding capacity of these epitopes was measured in vitro using purified HLA-A11 molecules (Table I). It was noted that all of them bound HLA-A11 with high affinity, in the 4 to 20 nM range. This finding is in agreement with previous studies in the A2.1 system (27, 28) indicating that ~90% of previously described epitopes had a high affinity (<50 nM) for HLA-A2.1.

Next, for each of the A11 epitopes, six individual A11/K^b mice were immunized s.c. at the base of the tail with 50 μg of the CTL epitope and 140 μg of a helper epitope (HBVc 128–140) emulsified in IFA. The I-A^b-restricted epitope, HBVc 128–140 (46), was used to ensure that adequate class II-restricted T cell help was available. After 11 days, the spleens were removed and restimulated in vitro with 1 μg/ml of the same CTL epitope used in the immunizations. Standard CTL assays were performed after 6 days in culture, using peptide-pulsed A11/K^b-transfected human EBV-transformed B lymphoblastoid cells (.221 A11/K^b) as target cells.

Representative responses from individual mice for each of the peptides are presented in Figure 2. All seven immunodominant A11 epitopes induced Ag-specific CTL capable of killing in the range of 40 to 70% at an E:T of ~50:1. To facilitate averaging and comparison of the responses from different animals and for different peptides, data were calculated in $LU_{30}/10^6$ effector cells, as described in *Materials and Methods*. A positive CTL response was defined as 2 or more $LU_{30}/10^6$ (28). Table I summarizes the CTL responses obtained with the seven immunodominant A11 epitopes. Positive CTL responses varied on average from about 5 $LU_{30}/10^6$ for HIV pol 325 and HIV pol 507 to approximately 30 $LU_{30}/10^6$ cells in the case of EBNA 416 and HIV nef 84.

To address the specificity of the observed CTL responses, we also immunized nontransgenic mice (C57BL/6J) with the same peptides. As expected, no CTL responses were observed when the same .221 A11/K^b cells used in the case of A11/K^b transgenic mice were used as target cells in the chromium assay (Table I). These data confirm that the CTL responses measured are specific and restricted by HLA-A11/K^b. Finally, it is of interest to note that the A11/K^b-restricted CTL responses described herein are comparable in magnitude with those obtained by immunizing normal C57BL/6 mice with the well-characterized potent immunogen $Ova_{257-264}$ (34 $LU_{30}/10^6$, data not shown).

Immunogenicity vs binding capacity for A11 motif-containing peptides

Previous studies (27, 28) by our group have examined the relationship between class I binding affinity and immunogenicity of A2.1-binding peptides in various experimental systems, including recall CTL responses from acute hepatitics, in vitro primary CTL responses from PBMC of normal human donors, and peptide immunizations in the A2.1/K^b transgenic mouse system. By analyzing the immunogenicity of potential epitopes ranging in binding affinity over a 10,000-fold range, it was determined that an A*0201 binding affinity threshold of approximately 500 nM was associated with immunogenicity.

To determine whether a similar affinity threshold exists in the A11/K^b transgenic system, the immunogenicity of a panel of 45 A11 motif (35–37)-containing peptides derived from various sources, including HBV, HCV, HIV, prostatic acid phosphatase (PAP), melanoma Ag (MAGE), EBNA, and *Plasmodium falciparum* Ags was examined. The binding affinities (IC_{50}) of the peptides comprising this panel ranged from 2 to 7500 nM.

Of the 28 A11 peptides that bound with high affinity (IC_{50} < 50 nM), 21 (75%) were found to elicit specific CTL in HLA-A11/K^b transgenic mice (Table IIA). By contrast, 54% (7 of 13) of the intermediate A11 binders (IC_{50} = 50–500 nM), were immunogenic, (Table II*B*). Finally, only 1 of 4 peptides demonstrated immunogenicity (Table II*C*) when binding capacity was >500 nM. These results are similar to those obtained in the case of A2.1/K^b mice (27, 28) in which 15 of 16 (94%) of the high binders, 8 of 21 (38%) of the intermediate binders, and 0 of 8 of the low binders were immunogenic. Taken together, these results indicate that the A11/K^b transgenic mouse system may provide a useful tool for the evaluation of the immunogenicity of putative A11-restricted epitopes.

Human vs murine CTL induction using A11 motif peptides

It has been reported that murine TAP molecules do not efficiently transport peptides with basic C termini (as those bound by HLA-A11). However, a functional CTL repertoire appeared to be generated in the A11/K^b transgenic mice, as demonstrated by the fact

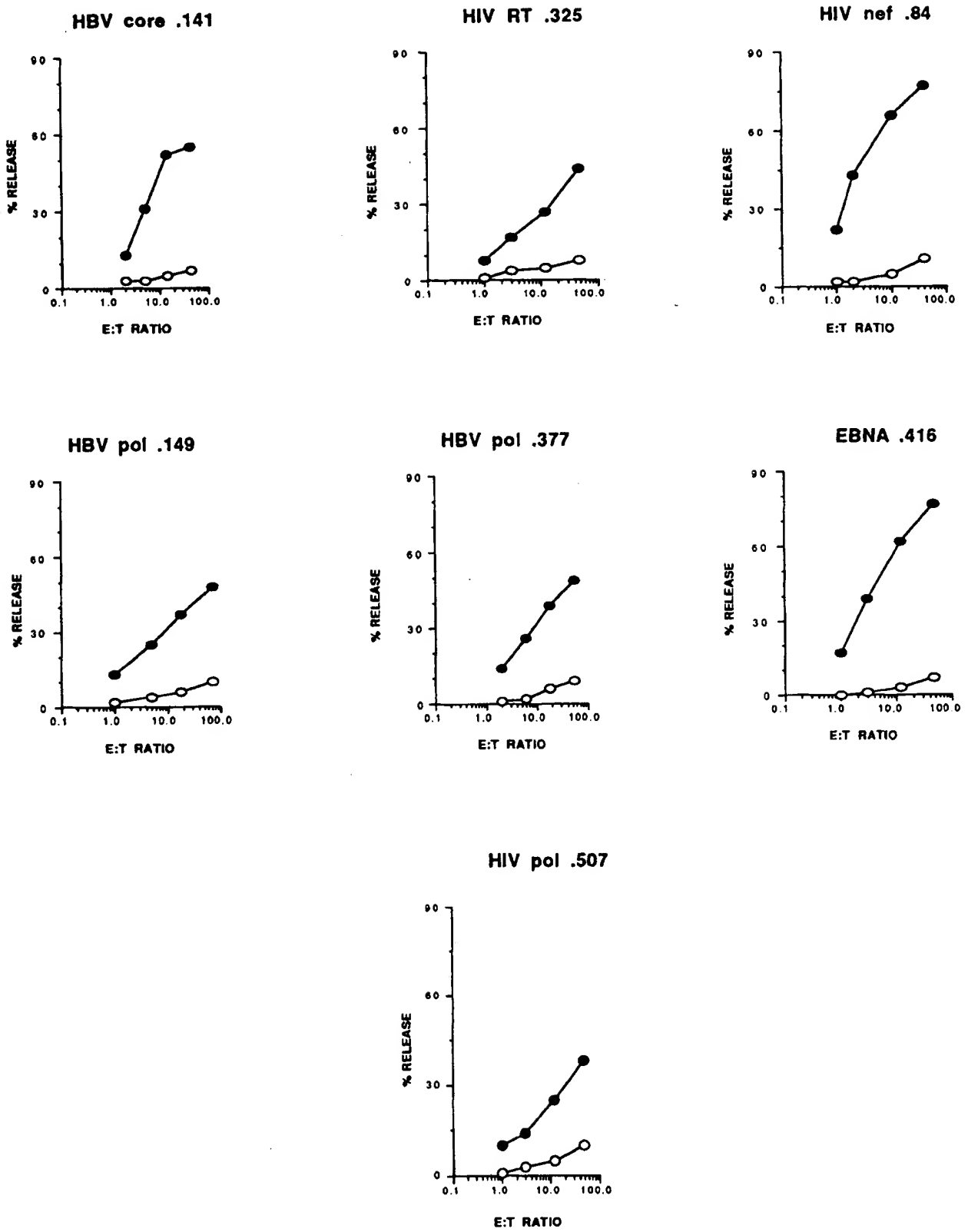

FIGURE 2. In vivo priming of HLA-A11/K^b transgenic mice. Splenocytes from peptide-primed transgenic mice were restimulated in vitro with peptide-coated LPS blasts. After 6 days, effector cells were assayed for CTL activity against ^{51}Cr-labeled .221 A11/K^b target cells in the absence (○) or presence (●) of peptide.

that 7 of 7 known A11-restricted epitopes were immunogenic in the A11/K^b transgenic mice. To further examine the extent of the overlap of the CTL repertoire in A11/K^b transgenic mice relative to humans expressing the HLA-A11 Ag, a set of 19 A11-binding peptides (IC_{50} in the 4–1200 nM range), previously tested (Table I and Table II) in the A11/K^b transgenic mice, were examined for

Table II. *Immunogenicity vs binding capacity of A11 motif peptides*

Peptide	Sequence	A11 Binding Capacity nM IC_{50}	CTL Response	
			No. Positive/No. Tested	$LU_{30}/10^6$ cells[a]
A. High binders				
HIV pol. .419	TVQPIVLPEK	2.4	2/9	2.0
HBV pol. 398	GSTHVSWPK	3.1	6/9	15.6 (1.5)
HIV pol. 1434	AVFIHNFKR	3.3	3/6	14.0 (1.3)
HIV env .49	TVYYGVPVWK	4.0	28/33	13.4 (3.1)
HBV core .141	STLPETTVVRR	4.0	6/6	12.1 (2.6)
HBV pol .110	IMPARFYPK	4.1	0/6	—[b]
HCV ns4 .1863	GVAGALVAFK	4.2	6/6	13.1 (1.4)
HIV pol .325	AIFQSSMTK	4.3	5/6	4.1 (1.7)
HIV nef .84	AVDLSHFLK	5.0	6/6	33.3 (1.4)
MAGE 2 .73	TTINYTLWR	5.5	6/6	57.7 (1.5)
HBV ADR × .1523	TTDLEAYFK	6.5	0/6	—
HIV pol .1434	AVFIHNFKRK	7.1	0/6	—
HIV pol .1225	KVYLAWVPAHK	7.6	6/6	16.1 (4.4)
HIV pol .1253	KVLFLDGIDK	7.7	6/6	30.4 (1.4)
HIV pol .919	QMAVFIHNFK	8.4	3/6	39.2 (1.1)
P. falciparum LSA1 .1855	LSTNLPYGK	8.6	6/6	51.4 (1.4)
HBV pol .654	QAFTFSPTYK	11	0/6	—
HIV env .2184	VTVYYGVPVWK	11	6/6	14.1 (1.3)
HBV pol .149	HTLWKAGILYK	14	6/6	13.1 (1.2)
HIV pol .1075	IVIWGKTPK	16	0/6	—
HBV pol .377	LVVDFSQFSR	17	6/6	10.1 (2.5)
HBV pol .150	TLWKAGILYK	17	0/6	—
HIV pol .438	ASQIYAGIK	18	6/6	25.3 (1.3)
EBNA .416	IVTDFSVIK	18	5/6	28.7 (4.8)
HIV pol .507	QIYQEPFKNLK	21	5/6	6.2 (2.3)
EBNA 4A .399	AVFDRKSDAK	22	6/6	59.1 (1.7)
HPV E6 .106	LURCNCQK	33	6/6	28.1 (1.4)
HIV pol .272	GIPHPAGLK	35	0/6	—
B. Intermediate binders				
HBV ADR pol .711	AVNHYFKTR	61	6/9	5.5 (2.2)
P. falciparum SSP2 .122	LSTNLPYGK	70	2/6	3.8
HBV ADR X .1548	KVFVLGGCR	73	0/6	—
HPV 16 E7 .88	GIVCPICSQK	90	3/9	13.9 (1.3)
HBV pol .47	NVSIPWTHK	105	0/6	—
HIV pol .1227	YLAWVPAHK	106	0/6	—
HBV pol .735	GTDNSVVLSR	107	3/9	13.1 (1.5)
PAP .274	ATQIPSYKK	128	6/6	38.6 (1.4)
HIV env .370	RAKWNNTLK	188	0/6	—
P. falciparum trap .511	LACAGLAYK	207	6/6	51.4 (2.0)
HBV ADR pol .601	RLADEGLNR	245	0/6	
HBV X .69	CALPFTSAR	316	7/9	30.1 (1.7)
HIV pol .859	MTKILEPFR	378	0/8	—
C. Low binders				
HBV pol .415	LSLDVSAAFY	545	0/6	—
MAGE 1 .95	ESLFRAVITK	1154	6/6	77.7 (1.3)
HIV-1 nL43 env GP .768	RLRDLLLIVTR	6667	0/6	—
HBV pol .149	HTLWKAGILY	7500	0/6	—

[a] Geometric mean of positive cultures (×/÷ SD).
[b] — Indicates no response detected.

their ability to induce primary in vitro CTL responses using human PBMCs.

It was found (Table III) that 8 of the peptides tested were able to elicit specific CTL responses. All of these peptides also had been shown to be immunogenic in HLA-A11/K^b transgenic mice. Of the remaining 11 peptides for which CTL responses were not observed in primary human in vitro cultures, 7 had generated specific CTL responses in HLA-A11/K^b transgenic mice, while the remaining 4 peptides were nonimmunogenic in both the human in vitro and the transgenic in vivo systems.

These data indicate that the CTL repertoire of HLA-A11/K^b transgenic mice is not limited and appears to be at least comparable, if not in fact greater, than that of HLA-A11 humans. It is noteworthy that the two known dominant human epitopes, HIV pol 325 and HIV pol 507, failed to generate CTL responses in the human primary in vitro system but scored positive in the A11/K^b transgenic mice. The observation that A11-binding peptides are less frequently immunogenic in the primary in vitro human assay than in the secondary HLA-A11/K^b transgenic murine assay is thus likely reflective of a relatively lower sensitivity of the in vitro primary human assay.

CTL induction in A11/K^b transgenic mice following immunization with whole influenza virus A/PR/8/34

To examine whether A11/K^b transgenic mice could recognize A11/K^b-restricted epitopes as a consequence of natural processing of complex Ags and presentation of the resulting peptides, we immunized HLA-A11 mice with whole influenza virus A/PR/8/34. A preliminary set of experiments was performed to identify potential influenza-derived epitopes. The sequences of hemagglutinin, matrix protein 1, and nuclear viral proteins were searched for the presence of a preferred HLA-A11 motif (35–37), a V, M, T, or

Table III. *HLA-A11 vs A11/K^b CTL responses*

Peptide	A11 Binding Capacity nM IC_{50}	CTL Response: Human No. Positive/ No. Tested	CTL Response: Human LU_{30}/ 10^6 cells[a]	CTL Response: Murine
HBV core .141	4.0	3/4	10.9 (7.0)	+
HCV ns4 .1863	4.2	5/6	47.1 (5.1)	+
HIV pol .325	4.3	0/4	—[b]	+
MAGE 2 .73	5.5	3/4	19.8 (11.8)	+
HIV pol .1434	7.1	0/4	—	—
hIV pol .1225	7.6	0/4	—	+
HIV pol .1253	7.7	0/4	—	+
HIV env .2184	11	3/4	15.6 (6.0)	+
HBV pol .149	14	0/4	—	+
HBV pol .377	17	0/4	—	+
HIV pol .507	21	0/4	—	+
HPV E6 .106	33	2/4	11.4 (2.9)	+
HIV pol .272	35	0/4	—	—
HPV 16 E7 .88	90	2/4	64.9 (21.1)	+
HBV pol .47	105	0/4	—	—
HIV pol .1227	106	0/4	—	—
HBV pol .735	107	0/4	—	+
PAP .274	128	2/2	96.3 (1.7)	+
MAGE 1 .95	1154	2/2	4.0 (1.0)	+

[a] Geometric mean of positive cultures (×/÷ SD).
[b] — Indicates no response detected.

I at position 2 and a K at the carboxyl terminus. Of 40 distinct 9-, 10-, and 11-mer peptides, 23 peptides bound A11 in vitro with an IC_{50} of 500 nM or less. Accordingly, these peptides were examined for immunogenicity in the HLA-A11/K^b transgenic mice. Eleven of them generated an immune response (at least two or more positive cultures of six tested) in A11/K^b transgenic mice (Table IV).

Subsequently, 3 wk after i.p. immunization of 100 to 300 hemagglutinating units of virus, splenocytes were stimulated in vitro with the 11 influenza-derived, potential A11 epitopes. As shown in Table IV, a brisk CTL response was detected for the hemagglutinin 458 peptide. Five of five mice immunized responded, with CTL responses averaging 14 $LU_{30}/10^6$ cells. By contrast, none of the remaining peptides elicited significant recall CTL responses in any of the cultures tested.

In conclusion, the ability of the hemagglutinin 458 peptide to expand a recall CTL response following immunization with whole influenza virus suggests that HLA-A11/K^b transgenic animals can, at least to some extent, process and present A11/K^b-restricted epitopes.

Discussion

In the present study, the derivation of HLA-A11/K^b transgenic mice is described. In this respect, it generalizes the previously reported generation of A2/K^b transgenic mice (26). In addition, novel observations relevant to our understanding of the role of TAP molecules in generation of a mature CTL repertoire were made. To facilitate the design of epitope-based vaccines that guarantee broadly based and non-ethnically biased population coverage, the development of HLA class I transgenic mice other than the well-described A2.1 line may be of particular importance. HLA-A11 is one of the most common class I HLA Ags, with phenotypic frequencies in the 4 to 33% range, depending on the particular ethnicity considered (33). Its binding motif has been described (35–37) and is characterized by a preference for peptides with small or hydrophobic residues (A, V, L, I, M, S, or T) in position 2, and positively charged C termini (R or K). Recent studies have suggested that HLA-A11 is part of a group of HLA Ags, designated the A3 supertype (38), that share similar binding motifs. Because of the high representation of the A3 supertype in most major ethnic groups considered, development of A11 transgenic mice is of potentially broad significance.

When we set out to generate HLA-A11-K^b transgenic mice, it was considered that certain aspects of the class I Ag-processing pathway, particularly differences of TAP specificity in molecules of mouse and human origin could potentially pose significant limitations to a successful outcome. Importantly, several studies suggested that because of the murine TAP specificity, peptides with C-terminal positive charges would not be efficiently transported (22–24), thereby potentially precluding formation of a functional CTL repertoire in the HLA-A11/K^b transgenic mice. However, it had been demonstrated that mouse mastocytoma cells (P815) transfected with HLA-A11 could be lysed by human A11-specific CTL (53) and that peptides with charged C termini could be eluted from HLA-A3 molecules expressed on P815 cells (54).

An additional concern was that TAP incompatibilities would result in reduced levels of surface class I molecules and peripheral $CD4^-CD8^+$ T cells, as has been demonstrated in studies of TAP-deficient mice (55, 56). However, it was subsequently noted that this reduced population of peripheral $CD4^-CD8^+$ T cells in TAP-deficient mice displayed similar Vβ usage relative to wild-type mice and, importantly, also exhibited peptide specificity and diversity (57).

Herein, we found good expression of HLA-A11/K^b molecules on T and B peripheral cells from the A11/K^b transgenic mice. The expression level was very similar to levels of HLA-A2.1 (approximately 135–160 MFC) detected in A2.1/K^b transgenic mice. It would, therefore, appear that the reported unfavorable specificity of murine TAP does not prevent stabilization and transport of HLA-A11/K^b molecules in transgenic mice in vivo.

In terms of whether the HLA-A11/K^b transgenic mice had a functional A11-restricted CTL repertoire, we initially examined the immunogenicity of various known HLA-A11-restricted epitopes. These epitopes were capable of generating CTL responses, with approximately 40 to 70% specific ^{51}Cr release at an E:T of ~50:1 (corresponding to 5–30 $LU_{30}/10^6$). The A11 specificity of the observed CTL responses was demonstrated by using as target cells in the ^{51}Cr release CTL assay the cell line A11/K^b.221, which expresses only the A11/K^b Ag and no other mouse or human class I Ag. In addition, when C57BL/6J mice were immunized with these same epitopes, no CTL response was observed when the A11/K^b.221 cells were used as targets.

To evaluate in more detail the overlap between the CTL repertoire of A11/K^b mice and humans expressing the A11 Ag, 19 peptides previously tested in the A11/K^b mice peptide immunization system were assayed for their ability to induce specific CTL in vitro using human PBMCs. It was found that all of the 8 peptides that were able to expand PBMC in vitro were also immunogenic in the murine system. Of the remaining 11 peptides, 7 generated specific CTL response in transgenic mice but not in primary human CTL cultures, while the remaining 4 peptides were found to be nonimmunogenic in both systems. This observation could suggest that transgenic mice might actually have a broader CTL repertoire than A11 humans. However, it is more likely that the human in vitro primary CTL assay is less sensitive than the mouse in vivo assay, as suggested by the observation that two known dominant epitopes from human patient studies, HIV pol 325 and HIV pol 507, failed to generate a human in vitro primary CTL response.

Taken together, the demonstration of immunogenicity for the A11-restricted peptides in the above scenarios suggests that the

Table IV. *A11-restricted influenza responses in HLA-A11/K^b transgenic mice*

Peptide	Position	Sequence	A11 Binding Capacity nM IC_{50}	CTL Response[a]			
				Peptide immunization		PR8 (FLU) immunization	
				No. Positive/ No. Tested	$LU_{30}/10^6$ cells[b]	No. Positive/ No. Tested	$LU_{30}/10^6$ cells
Hemagglutinin							
	.148	GVTAACSHAGK	30	3/6	11.8 (2.4)	0/5	—[c]
	.168	LTEKEGSYPK	121	3/6	11.8 (2.0)	0/5	—
	.314	PVTIGECPK	2.6	2/6	4.3 (1.5)	0/5	—
	.458	NVKNLYEKVK	51	6/6	10.3 (1.8)	5/5	14.0 (2.2)
Matrix 1							
	.130	SIIPSGPLK	44	6/6	7.1 (2.3)	0/5	—
	.470	KTRPILSPLTK	0.7	6/6	24.3 (2.0)	0/5	—
	.179	MVLASTTAK	169	3/6	90.1 (1.3)	0/5	—
	.243	RMGVQMQRFK	94	6/6	23.5 (1.3)	0/5	—
Nuclear protein							
	.220	ATEIRASVGK	75	3/6	12.9 (1.2)	0/5	—
	.189	MVMELVRMIK	193	6/6	18.9 (2.0)	0/5	—
	.349	GTKVVPRKG	72	3/6	3.5 (1.6)	0/5	—

[a] Peptide-pulsed .221 A11/Kb target cells.
[b] Geometric mean of positive cultures (×/÷ SD).
[c] — Indicates no response detected.

CTL repertoire in HLA-A11 humans and the HLA-A11/K^b transgenic mice may be quite similar. This is somewhat surprising in that murine TAP has been reported not to transport A11-restricted peptides efficiently. It is likely that this inefficient transport is sufficient to load and stabilize HLA-A11/K^b to the extent of driving thymocyte education. Other possibilities include TAP-independent transport or trimming of peptides after transport (58–61).

We also immunized these A11/K^b transgenic mice with whole influenza virus, followed by in vitro stimulation with the A11-binding influenza-derived potential epitopes. Indeed, one peptide was able to recall a specific influenza A11/K^b-restricted CTL response. These results indicated that A11/K^b transgenic animals can, at least to some extent, generate A11-restricted epitopes as a result of natural Ag processing. Specifically, while it is clear that hemagglutinin 458 is recognized well by the transgenic mouse CTL, it is not clear that this peptide truly represents an authentic epitope for human CTL, which is important to strengthen the contention that the repertoire in these transgenic mice resembles that of the A11 human. Future studies will address this issue in more detail by comparing CTL responses induced by influenza infection of A11/K^b transgenic mice relative to those generated in humans expressing the HLA-A11 Ag.

To examine the potential usefulness of the A11/K^b mice in terms of epitope identification and compare them with the previously described A2.1/K^b transgenic mice, we have examined the immunogenicity of a panel of 45 A11 motif peptides in which the A11-binding capacity (IC_{50}) ranged from 2 to 7500 nM. These peptides were derived from various sources, including HIV, HBV, HCV, EBNA, PAP, MAGE, and *P. falciparum*. It was found that 75% (21 of 28) of the peptides with high binding capacity (IC_{50} < 50 nM) and 54% (7 of 13) of the peptides with intermediate binding capacity (IC_{50}, 50–500 nM) were able to induce a CTL response. These results are similar to results obtained in the A2.1/K^b transgenic system (27, 28) in which 94% (15 of 16) high capacity binders and 38% (8 of 21) of intermediate binders were found to be immunogenic. Thus, using a binding threshold of 500 nM, and given an equal number of intermediate and high binding peptides, ~65% of the peptides would score immunogenic in the two HLA transgenic systems. This similar pattern of immunogenicity observed would suggest that HLA-A11/K^b mice may have utility for the identification of human CTL epitopes, as has been demonstrated in the case of HLA-A2.1/K^b mice (26, 28, 30).

In conclusion, we report the derivation of HLA-A11/K^b transgenic mice. These mice are endowed with a functional A11/K^b-restricted CTL repertoire. Therefore, this system may be used as an immunologic tool for epitope identification and vaccine development. In addition, these mice will be of potential utility in studies analyzing the influence of TAP specificity on CTL repertoire generation and the mechanisms involved in Ag processing and presentation.

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TAP (Transporter Associated with Antigen Processing)-Independent Presentation of Endogenously Synthesized Peptides Is Enhanced by Endoplasmic Reticulum Insertion Sequences Located at the Amino- but not Carboxyl-Terminus of the Peptide

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Under most circumstances, cell surface MHC class I molecules display peptides derived from a cytosolic pool of proteins. The efficient presentation of such peptides requires the functioning of two MHC gene products [TAP1 and TAP2 (transporter-associated with Ag processing 1 and 2)] that form a complex that facilitates transmembrane movement of peptides from the cytosol to the endoplasmic reticulum, the site of peptide association with class I molecules. It has been previously shown that peptides can be presented in a TAP-independent manner in association with HLA A2.1 or H-2 K^d if they are expressed COOH-terminal to an endoplasmic reticulum insertion/signal sequence derived from the adenovirus E3/19K glycoprotein (Anderson et al., 1991. *J. Exp. Med. 174: 489*; Eisenlohr et al., 1992. *Cell 71: 963*). We show that: 1) the E3/19K signal sequence greatly enhances the presentation of each of four additional peptides tested in association with H-2 K^b or K^k, 2) the E3/19K signal sequence can be substituted by a signal sequence derived from β-IFN, and 3) the E3/19K signal sequence does not function when located at the COOH terminus of antigenic peptides. These findings indicate that first, many peptides require TAP for efficient presentation to T cells, second, expression of peptides COOH-terminal to signal sequences is a generally applicable method of bypassing the TAP-dependence of peptide presentation and third, the leader sequence does not act to bypass TAP simply by increasing the hydrophobic nature of peptides. *Journal of Immunology,* 1994, 152: 381.

MHC class I molecules function to transport peptides of 8 to 10 residues to the cell surface where they can be perused by T_{CD8+}[2] (1–3). The association of antigenic peptides with class I molecules occurs in an early secretory compartment, probably the ER (4–6). In most cases, peptides are derived from proteins located in the cytosol (7, 8). The efficient presentation of cytosolic Ag depends on the function of two MHC-encoded proteins (TAP1 and TAP2) that belong to a family of proteins whose members transport various substances (ranging from ions to proteins) across membranes (9). TAP1 and TAP2 form TAP, which facilitates peptide binding to class I molecules (10–15). Direct evidence for TAP-mediated transport of peptides into the ER has recently been published (16, 17).

Class I molecules derived from TAP-deficient cells are not always completely devoid of peptides. HLA A2.1 molecules derived from TAP-deficient cells contain large amounts of peptides derived from ER insertion/signal sequences (18, 19). These hydrophobic sequences (henceforth referred to as ER insertion sequences) are usually located at the extreme NH_2-terminus of proteins, and are efficiently cleaved by signal peptidase from proteins during the process of protein translocation into the ER.

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[2] Abbreviations used in this paper: T_{CD8+}, $CD8^+$ T lymphocytes; BFA, brefeldin A; ER, endoplasmic reticulum; HA, hemagglutinin; N, nucleocapsid protein; NP, nucleoprotein; OVA, ovalbumin; PR8, influenza virus A/Puerto Rico/8/34; rVV, recombinant vaccinia virus; TAP, transporter associated with Ag processing; VSV, vesicular stomatitis virus; SRP, signal recognition particle.

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HLA A2.1 molecules from TAP-deficient cells were shown to efficiently present a biosynthesized determinant from influenza virus M1 protein located COOH terminal to the adenovirus E3/19K glycoprotein ER insertion sequence (20). Subsequently, it was shown that H-2 K^d molecules expressed in TAP-deficient cells also efficiently present a determinant from influenza virus nucleoprotein located COOH terminal to the same ER insertion sequence (21). In both cases, presentation of the biosynthesized peptide without the ER insertion sequence was low or nondetectable.

It appears then that ER insertion sequences provide an alternative means for cytosolic peptides to gain access to newly synthesized class I molecules. This route is potentially of practical importance in as much as it might provide a more efficient method of loading class I molecules with antigenic peptides in vivo. We characterize the mechanism by which ER insertion sequences bypass the requirement for TAP in presentation of biosynthesized peptides by examining the presentation to T_{CD8+} of various peptide-ER insertion sequence chimeric gene products produced by TAP expressing and TAP-deficient cells.

Materials and Methods

Cells and virus

T2 cells (22), T2 cells transfected with H-2 K^k (T2 K^k) or K^b (T2 K^b), and C1R cells transfected with K^k (C1R K^k) were provided by Dr. P. Cresswell (Yale University, New Haven, CT). T2 cells and transfectants were maintained in IMDM supplemented with 7.5% (v/v) FBS. L929 cells were maintained in DMEM supplemented with 7.5% (v/v) FBS. PR8 (H1N1) and Sendai virus were grown in 10-day-old embryonated chicken eggs and were used as infectious allantoic fluids. VSV was grown in baby hamster kidney cells. rVV were propagated in thymidine kinase-deficient human 143B osteosarcoma cells. VV-NP and VV-N have been described (23, 24). rVV expressing H-2 K^d (VV-K^d) and K^b were created as described (25) using plasmids created by inserting cDNA encoding the respective genes behind the early/late VV p7.5 promoter into pSC11 plasmid modified to contain a multiple cloning site downstream of the p7.5 promoter. rVV expressing minigenes were similarly produced by inserting oligonucleotides corresponding to the appropriate sequence into the modified version of pSC11. rVV expressing minigenes COOH-terminal to the E3/19K leader sequence were created by inserting the appropriate synthetic oligonucleotide into a pSC11 modified to contain a sequence downstream of the p7.5K promoter encoding the E3/19K leader sequence, ending with a *NotI* site and an appended *StyI* site (21). This resulted in the insertion of an additional Ala between the leader and the antigenic peptide. rVV expressing minigenes amino terminal to the E3/19K leader sequence were created by inserting the appropriate synthetic oligonucleotide into a pSC11 modified to contain a sequence encoding the E3/19K leader sequence preceded immediately upstream by a *NdeI* site, and seven bases further upstream by a *SalI* site. After digestion with *NdeI* (partial) and *SalI*, synthetic oligonucleotides corresponding to the appropriate antigenic peptide sequence and containing $\overset{CCACCATG}{GGTGGTAC}$ immediately 5′ to the peptide-coding sequence were ligated to the plasmid. This resulted in the restoration of the first Met of the E3/19K signal peptide. A rVV expressing the $NP_{147-155}$ peptide COOH-terminal to the β-IFN ER signal peptide was produced from a plasmid generously provided by Dr. Laurence Eisenlohr (Thomas Jefferson University, Philadelphia, PA) that will be described in another publication. The designations and peptide products of the various rVV are given in Table I. Where indicated, rVV were purified by centrifuging 1 ml of crude infectious stocks for 20 min at 500,000 × *g*, suspending the pellet in 200 μl basal salt solution supplemented with 0.1% BSA (BSS-BSA) and recentrifuging through 2 ml of 24% sucrose. Pellets were then resuspended in 600 μl BSS-BSA and stored overnight at 4°C before use.

Table I. *Foreign gene products produced by rVV*[a]

Designation	Sequence of Gene Product
VV-NP	495 residue protein
VV-$NP_{M147-155}$	*M*TYQRTRALV
VV-ES $NP_{147-155}$	**MRYMILGLLALAAVCSA***A*TYQRTRALV
VV-$NP_{M147-155}$ ES	*M*TYQRTRALV**MRYMILGLLALAAVCSA***A*
VV-IS $NP_{147-155}$	**MTNKCLLQIALLLCFSTTALS**TYQRTRALV
VV-NP_{M50-57}	*M*SDYEGRLI
VV-ES NP_{50-57}	**MRYMILGLLALAAVCSA***A*SDYEGRLI
VV $OVA_{M257-264}$	*M*SIINFEKL
VV $ESOVA_{M257-264}$	**MRYMILGLLALAAVCSA***A*SIINFEKL
VV $OVA_{M257-264}$ ES	*M*SIINFEKL**MRYMILGLLALAAVCSA**
VV-N_{M52-59}	*M*RGYVYQGL
VV-ES N_{52-59}	**MRYMILGLLALAAVCSA***A*RGYVYQGL
VV-N_{M52-59} ES	*M*RGYVYQGL**MRYMILGLLALAAVCSA***A*
VV-$SNP_{M321-328}$	*M*APGNYPAL
VV-ES $SNP_{321-328}$	**MRYMILGLLALAAVCSA***A*APGNYPAL
VV-$SNP_{M321-328}$ ES	*M*APGNYPAL**MRYMILGLLALAAVCSA***A*

[a] *Italics*, foreign initiating methionine; Underlined, antigenic peptide P; **Bold**, ER insertion sequence; *Underlined italic*, additional Ala resulting from inclusion of a convenient restriction site in the expression vector.

Mice

The 6- to 8-wk-old C57BL/6 (H-2^b), and BALB/c (H-2^d) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were immunized with PR8, VSV, or Sendai virus by i.p. injection, and with rVV by i.v. injection.

Cytotoxicity assays

Target cells were infected with viruses as described previously (21, 23). To generate T_{CD8+}, splenocytes derived from animals immunized with viruses 2 to 6 wk previously were stimulated in vitro for 6 days with either antigenic peptides at 1 μg/ml or with virus infected-autologous splenocytes as described (23). Synthetic peptides were provided by the Biological Resources Branch, NIAID. Microcytotoxicity assays were performed at previously described (6, 23), with the exception that target and effector cells were incubated for 6 h instead of 4 h. Data are expressed as percent specific release defined as: ((experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)) × 100.

Results

Description of experimental system

All of the experiments described below use the T2 cell line. These cells have a single copy of chromosome 6 derived from 721.174 cells, a γ-irradiated mutagenized EBV-transformed lymphoid cell line selected for low cell surface expression of class I molecules (26). Irradiation induced a 1 Mbp deletion in the HLA class II region of this chromosome. Within this region are the genes encoding TAP1 and TAP2. The loss of this region results in the diminished assembly and transport of class I molecules, and the inability of cells to present determinants derived from cytosolic proteins (10–15). Class I folding, transport, surface expression, and peptide presentation are restored to normal levels by introduction of the TAP genes, which indicates that the absence of TAP genes is the major, if not the sole, reason for the deficit in class I assembly and Ag presentation (27, 28).

We recently demonstrated that human cells infected with rVV encoding mouse class I α-chains can be recognized by mouse T_{CD8+} (29, 30). By coinfecting human

cells with rVV encoding mouse class I α-chains and a viral gene product it is possible to test presentation of defined peptides known to be recognized by mouse T_{CD8+}. The T2 cell line, like most EBV-transformed cell lines, is able to at least partially support a VV infection, and expresses both early and late viral gene products. In what follows, we have used rVV to assess the ability of T2 cells to present determinants from OVA, VSV, influenza virus, and Sendai virus to mouse T_{CD8+}. In all cases, control experiments that are not illustrated verified the need for expression of the appropriate mouse class I molecule. In each experiment shown but one, mouse L929 (H-2^k) cells are used as examples of Ag processing competent cells to demonstrate the integrity of rVV expressing Ag not presented by T2 cells. As with T2 cells, control experiments verified the need for rVV directed expression of the appropriate mouse class I molecule. Results similar to those obtained with L929 cells were obtained using nonmutant human EBV-transformed cells (not shown), or with 0.174 cells transfected with genes encoding TAP1 and TAP2 (not shown).

E3/19K ER insertion sequence facilitates presentation of K^b and K^k-restricted peptides

We initially examined the capacity of the E3/19K leader sequence to facilitate presentation of K^b restricted peptides from Sendai virus NP (31), VSV N (1), and OVA (32). Six rVV were constructed that express under control of the viral p7.5 early/late promoter the determinant preceded by an initiating Met, or the determinant preceded by the E3/19K ER insertion sequence (the sequences of the peptides produced by rVV and their designations are summarized in Table I). For VSV N and OVA, the determinants corresponded to the naturally processed peptides, which optimally bind K^b. For Sendai virus NP, the determinant produced by the rVV is missing the amino terminal Phe that is likely present in the naturally processed peptide, because at the time this study was initiated, it was thought that the naturally processed peptide was a octamer, like previously characterized K^b-restricted peptides (33, 34).

The recombinants were tested for their abilities to sensitize L929 cells or T2 cells for lysis by secondary in vitro-stimulated mouse T_{CD8+} populations raised to the determinant or full length protein containing the determinant. As seen in Table II, each of the recombinants was able to sensitize L929 cells for lysis by the appropriate T_{CD8+}. The leader sequence did not greatly affect presentation of determinants from VSV N or OVA but did enhance presentation of the Sendai virus minigene. Possibly the relatively poor recognition of cells infected by VV-$SNP_{M321-328}$ is related to the deletion of Phe. In T2 cells, presentation of leaderless determinants ranged from undetected (VSV N), to low but clearly above background levels (OVA). In each case presentation was greatly enhanced by addition of the E3/19K ER insertion peptide to the amino terminus of the peptide. Incidentally, note also that the leader sequence enhanced the degree of cross-recognition of the Sendai NP determinant by anti-N T_{CD8+}. Given the difficult task of the TCR in discriminating between highly similar surfaces presented for recognition (35, 36), it is perhaps only surprising that there is not more cross-reactivity among the various K^b-restricted populations. The enhanced cross-recognition observed with anti-N T_{CD8+} and ES-$SNP_{321-328}$ could reflect either quantitative or qualitative differences between the K^b-peptide complexes produced by cells infected with VV ES-$SNP_{321-328}$ and VV $SNP_{M321-328}$.

To further generalize the function of the E3/19K ER insertion sequence in bypassing TAP, we produced another pair of rVV encoding the K^k-restricted determinant from PR8 NP (residues 50–57) preceded by Met or the E3/19K ER insertion peptide. Again, the E3/19K ER insertion peptide facilitated the presentation of the viral peptide in T2 cells (Table III).

Based on these findings and our previous findings (21) we make two conclusions. First, each of the five minimal determinants (with an initiating Met) tested from viral proteins are presented in a TAP-dependent manner. Second, in each case, TAP-independent presentation is greatly enhanced by addition of the E3/19K ER insertion sequence to the amino terminus of the peptide.

Table II. *NH_2-terminal E3/19K ER insertion sequence facilitates presentation of K^b-restricted peptides*[a]

Target Cells	Percent Specific ^{51}Cr Release		
	Anti-VSV	Anti-Sendai	Anti-OVA
L929			
VV-K^b	16	6	11
VV-K^b + VV-N_{M52-59}	30	0	0
VV-K^b + VV-ES N_{52-59}	35	0	0
VV-K^b + VV-$SNP_{M321-328}$	4	22	0
VV-K^b + VV-ES $SNP_{321-328}$	52	58	8
VV-K^b + VV-$OVA_{M257-264}$	14	4	74
VV-K^b + VV-ES $OVA_{257-264}$	19	3	56
T2			
VV-K^b	11	4	4
VV-K^b + VV-N_{M52-59}	9	1	4
VV-K^b + VV-ES N_{52-59}	52	1	4
VV-K^b + VV-$SNP_{M321-328}$	3	9	6
VV-K^b + VV-ES $SNP_{321-328}$	23	52	2
VV-K^b + VV-$OVA_{M257-264}$	4	2	15
VV-K^b + VV-ES $OVA_{M257-264}$	4	1	70

[a] L929 or T2 K^k cells infected for 5 h with rVV were tested for lysis by secondary in vitro stimulated splenocytes in a ^{51}Cr release assay. VSV N-specific T_{CD8+} were induced by priming mice with VV-N and restimulating with VSV, and were used at an E:T ratio of 40:1. Sendai NP-specific T_{CD8+} were induced by priming mice with VV-ES $NP_{321-328}$ and restimulating with Sendai virus and were used at an E:T of 5:1. OVA-specific T_{CD8+} were induced by priming mice with VV-$OVA_{M257-264}$ and restimulating with the synthetic peptide SIINFEKL and were used at an E:T of 20:1. A similar pattern of reactivity was observed in the same experiment for each of the effector populations over a 27-fold E:T range.

Table III. *NH_2 terminal leader sequence facilitates presentation of K^k restricted peptide*[a]

Target Cells	Percent Specific ^{51}Cr Release by NP Peptide-Specific T_{CD8+} 6:1	2:1
C1R K^k		
VV	8	3
VV-NP_{M50-57}	18	14
VV-ES NP_{50-57}	24	18
T2 K^k		
VV	1	0
VV-NP_{M50-57}	0	0
VV-ES NP_{50-57}	44	22

C1R K^k or T2 K^k cells infected for 5 h with rVV were tested for lysis by secondary in vitro stimulated splenocytes in a ^{51}Cr release assay.

[a] PR8 NP-specific T_{CD8+} were induced by priming mice with NP-VV and restimulating with the synthetic peptide SDYEGRLI, and were used at the indicated E:T.

Table IV. *Peptide presentation is facilitated in T2 cells by a different ER insertion sequence*[a]

Target Cells	Percent Specific ^{51}Cr Release by NP Peptide-Specific T_{CD8+} 13:1	4:1
L929		
VV-K^d	16	13
VV-K^d + VV-NP	39	17
VV-K^d + VV-$NP_{M147-155}$	63	23
VV-K^d + VV-ES $NP_{147-155}$	77	50
VV-K^d + VV-$NP_{M147-155}$ ES	90	48
VV-K^d + VV-IS $NP_{M147-155}$	66	36
T2		
VV-K^d	0	0
VV-K^d + VV-NP	0	0
VV-K^d + VV-$NP_{M147-155}$	0	0
VV-K^d + VV-ES $NP_{147-155}$	47	35
VV-K^d + VV-$NP_{M147-155}$ ES	9	3
VV-K^d + VV-IS $NP_{M147-155}$	38	16

[a] L929 or T2 K^k cells infected for 5 h with rVV were tested for lysis by secondary in vitro-stimulated splenocytes in a ^{51}Cr release assay. PR8 NP-specific T_{CD8+} were induced by priming mice with NP-VV and restimulating with the synthetic peptide TYQRTRALV, and were used at the indicated E:T.

β-IFN leader sequence facilitates presentation of NP 147–155 determinant

The conclusion that ER insertion sequences are able to bypass the requirement for TAP in peptide presentation is based on results with only a single ER insertion sequence. To examine the generality of this finding, we examined the ability of a ER insertion sequence derived from β-IFN to facilitate T2 cell presentation of the K^d-restricted determinant from PR8 NP (residues 147–155) (Table IV). As we reported previously (21), although NP-specific T_{CD8+} do not lyse T2 cells coinfected with rVV expressing K^d and the 147–155 determinant, they lyse cells coexpressing K^d with ES $NP_{147-155}$. Similarly, T2 cells coexpressing K^d with IS $NP_{147-155}$ are lysed by NP-specific T_{CD8+}. As expected, L929 cells could also present the 147–155 determinant from IS $NP_{147-155}$. Thus, we conclude that the ability of the E3/19K ER insertion sequence to bypass the need for TAP is not unique, and applies to at least one, and probably many other ER insertion sequences.

Table V. *ER insertion sequence containing peptides sensitize cells in an intracellular compartment*[a]

T2 Target Cells	Percent Specific ^{51}Cr Release by NP or N-Specific T_{CD8+} E:T 1	E:T 2
Expt. A		
VV-K^d	1	1
VV-K^d + VV-ES $NP_{147-155}$ purified	56	39
VV-K^d + VV-IS $NP_{147-155}$ purified	55	45
VV-K^d + 5X unlabeled T2 VV-ES $NP_{147-155}$	14	13
VV-K^d + 5X unlabeled T2 VV-IS $NP_{147-155}$	8	9
Expt. B		
VV-K^b	1	1
VV-K^b + VV-ES N_{52-69} purified	56	39
VV-K^b + 3X unlabeled T2 VV-ES N_{52-59}	14	13

[a] In experiment A, target cells infected with crude VV-K^d and purified ES-rVV for 5 h were tested for lysis by secondary T_{CD8+} induced by VV-NP priming and in vitro stimulation with PR8 at E:Ts of 30:1 and 10:1. ^{51}Cr labeled VV-K^d cells were also tested for lysis in the presence of five times their number of unlabeled cells infected with VV-ES $NP_{147-155}$ or VV-IS $NP_{147-155}$. Experiment B is similar with the exception that cells were infected with VV-K^b and tested for recognition at E:Ts of 45:1 and 5:1 by secondary T_{CD8+} induced by VV-N priming and in vitro stimulation with VSV and synthetic peptide RGYVYQGL. ^{51}Cr labeled VV-K^b cells were also tested for lysis in the presence of three times their number of unlabeled cells infected with VV-ES N_{52-59}.

Endogenously synthesized ER-insertion sequence peptides sensitize cells for lysis in intracellular compartment

Antigenic peptides are generally only recovered from cells as part of the class I-peptide complexes. It was possible, however, that the delivery of antigenic peptide to the ER by ER insertion sequences resulted in the sequestration of peptides in the secretory compartment, where they might persist. Because the rVV stocks that we use are homogenates prepared from infected cells, it was possible that the stocks themselves contained antigenic peptides.

To examine this possibility we tested the ability of purified rVV to sensitize T2 cells for T_{CD8+} lysis. As seen in Table V, purification of VV-ES-$NP_{147-155}$, VV-IS-$NP_{147-155}$, or VV-ES-N_{52-59} did not adversely affect their capacity to sensitize VV-K^d- or VV-K^b-infected T2 cells for lysis by the appropriate T_{CD8+}. Furthermore, a high speed supernatant of virus stock depleted of ES-N_{52-59} virions did not sensitize VV-K^b-infected cells for lysis by N-specific T_{CD8+} (not shown). These findings indicate that sensitization by ER insertion sequence fusion peptides cannot be attributed simply to the presence of antigenic peptides in the virus preparation. It remained possible,

however, that sensitization was due to secretion of peptides during the course of target cell preparation and microcytotoxicity assay that then bound to class I molecules present on the cell surface. This possibility was tested by incubating labeled VV-K^d-infected T2 cells with five times their number of unlabeled VV-ES-$NP_{147-155}$- or VV-IS-$NP_{147-155}$-infected T2 cells during the course of a 6-h microcytotoxicity assay (Table V*A*). This resulted in a slight degree of lysis of the labeled T2 cells. Similar results were obtained with VV-ES N_{52-59} (Table V*B*). These findings indicate that small amounts of antigenic peptides are probably secreted by cells producing ER insertion sequence-fusion peptides, but that the bulk of sensitization is due to complexes formed intracellularly.

Position of ER insertion sequence is critical to its function

We next sought to determine whether the orientation of the leader sequence influences its ability to facilitate peptide presentation in T2 cells. Initially, we produced a rVV in which the $NP_{M147-155}$ minigene was fused at its COOH-terminus to the E3/19 ER insertion sequence. Although L929 cells infected with this rVV were lysed at or above levels observed with ES-$NP_{M147-155}$, T2 cells infected with $NP_{M147-155}$ ES were only marginally lysed above background levels (Table IV). This partial effect of the leader sequence in the COOH-position in facilitating TAP-independent presentation was observed in a number of experiments. Next, similar recombinants were produced using the three K^b-restricted viral determinants described above (Table VI). Again, each was able to sensitize L929 cells for lysis by the appropriate T_{CD8+}, demonstrating the integrity of the determinant. Despite this, the recombinants demonstrated little or no ability to sensitize T2 cells for T_{CD8+} lysis. Thus, we conclude that the position of the leader sequence relative to the class I-restricted determinant is critical to its ability to facilitate presentation in TAP-deficient cells.

Table VI. *ER insertion sequence must be placed at NH_2-terminus of antigenic peptides to facilitate presentation in T2 cells*[a]

Target Cells	Percent Specific ^{51}Cr Release by Peptide-Specific T_{CD8+} E:T 1	E:T 2
L929		
VV-K^b + VV-N_{M52-59}	25	8
VV-K^b + VV-ES N_{52-59}	36	24
VV-K^b + VV-N_{M52-59} ES	48	42
VV-K^b + VV-$SNP_{M321-328}$	21	18
VV-K^b + VV-ES $SNP_{321-328}$	48	25
VV-K^b + VV-$SNP_{M321-328}$ ES	40	14
VV-K^b + VV-$OVA_{M257-264}$	45	21
VV-K^b + VV-ES $OVA_{257-264}$	45	16
VV-K^b + VV-$OVA_{M257-264}$ ES	42	20
T2		
VV-K^b + VV-N_{M52-59}	0	0
VV-K^b + VV-ES N_{52-59}	39	31
VV-K^b + VV-N_{M52-59} ES	0	0
VV-K^b + VV-$SNP_{M321-328}$	9	5
VV-K^b + VV-ES $SNP_{321-328}$	54	30
VV-K^b + VV-$SNP_{M321-328}$ ES	14	9
VV-K^b + VV-$OVA_{M257-264}$	9	4
VV-K^b + VV-ES $OVA_{257-264}$	47	31
VV-K^b + VV-$OVA_{M257-264}$ ES	3	7

[a] These data were obtained in the same experiment as those shown in Table II, using the identical splenocyte populations. VSV N-specific splenocytes were used at E:T of 14:1 and 4:1, Sendai virus NP-specific splenocytes were used at E:T of 16:1 and 1:1, OVA-specific splenocytes were used at E:T of 7:1 and 2:1. Levels of specific ^{51}Cr release obtained against cells infected with VV-K^b only have been subtracted from each value. For VSV-, Sendai virus-, and OVA-specific populations with L929 cells these values are, respectively, at the higher E:T, 6%, 11%, and 5%, and at the lower E:T 5%, 3%, and 2%. For VSV-, Sendai virus-, and OVA-specific populations with T2 K^k cells these values are respectively at the higher E:T, 5%, 5%, and 3% , and at the lower E:T 5%, 2%, and 1%.

Discussion

We show that T2 cells demonstrate greatly compromised presentation to mouse T_{CD8+} of each of five signal-less endogenously synthesized peptides tested. This finding is in apparent conflict with those of Zweerink et al. (37) that T2 cells are able to present to human T_{CD8+} 4 different signal-less peptides produced from transfected minigenes. There are several potential reasons for this discrepancy. First, there obviously are chemical differences between the peptides examined in the two studies. This seems unlikely, however, to account for the observed differences because all of the HLA-restricted peptides were presented reasonably well, and perhaps more cogently, Zweerink et al. (37) noted that there is no simple correlation between the hydrophobicity and degree of presentation of the HLA-restricted peptides. Second, the transient expression of peptides via VV might disfavor presentation relative to constitutive expression from transfected genes, particularly if the transport of peptides from the cytosol occurs very slowly, or if the transfected genes direct the synthesis of greater quantities of peptides. Third, human T_{CD8+} might simply be more efficient at detecting class I peptide complexes on T2 cells than mouse T_{CD8+}. We marginally favor this latter possibility, because the interaction of adhesion molecules between T_{CD8+} and target cells is likely to be compromised in a heterologous mouse-human combinations (38). Indeed, it could be that additional interaction provided by adhesion molecules accounts for observations that RMA/S cells that are thought to be completely deficient in TAP2 expression (39) present Ag to mouse T_{CD8+} (40–42) although T2 and other TAP-deficient human cells by and large, do not.

We observed that addition of ER-insertion sequences to the amino termini of peptides greatly enhanced their presentation to T_{CD8+}. Similarly, Zweerink et al. (37) observed that the addition of ER insertion sequence to the

NH_2-terminus of peptides that were presented in the absence of the targeting sequence increased the amount of peptide delivered to HLA molecules as measured by increased difficulty to block with an anti-HLA mAb, and enhanced expression of cell surface class I molecules (37). Thus, although natural processed determinants can have the capacity to gain access to class I molecules in a TAP-independent manner, their delivery is clearly enhanced by addition of ER targeting sequence.

Importantly, we found that the ER insertion sequence must be placed at the amino terminus of the peptide to most efficiently enhance presentation, which indicates that the ER insertion sequence does not act solely by increasing the hydrophobicity of the peptide. There are several possible explanations for the difference between the antigenicity of the NH_2- and COOH-terminal ER insertion sequence fusion peptides: 1) Delivery of peptides to the ER might require transit through the protein conducting channel (43), and this might require the leader to be located at the NH_2-terminus; 2) the insertion sequence in the carboxyl-terminal position might also direct the antigenic peptide to the protein conducting channel, but signal peptidase is either unable to liberate the antigenic peptide or the peptide remains on the cytosolic side of the membrane; 3) the ER insertion sequence by virtue of its hydrophobicity might deliver peptides across the ER membrane in a channel-independent manner, but could only be cleaved from the peptide by signal peptidase (or possibly other ER peptidases) when present in the amino terminal position.

In considering these alternatives, it is instructive to review basic findings regarding the export of proteins into the ER. It is believed that for most proteins exported into the ER, SRP binds cotranslationally to nascent proteins as they are extruded from the ribosome. Binding of SRP slows translation until it associates with docking protein associated with the protein conducting channel. Translation then accelerates and the growing chain is extruded into the ER. Due to steric interference from the ribosome, the signal recognition particle generally binds to the signal only after the protein is 70 residues in length (44–47). Inasmuch as the ES peptide constructs are 27 to 28 residues, this mechanism cannot operate. It has been demonstrated, however, that signal peptides can direct proteins to the ER in a SRP-independent manner as long as the peptide maintains an extended structure (48–50), which is very likely to pertain to the ES peptides we have produced. Moreover, it has also been found that signal peptides can function even when located at a considerable distance from the amino terminus, as long as they are not buried in the interior of the protein after folding (51). Thus, it is likely that the peptide-ES constructs are also translocation competent. Results with other proteins indicate that the residues C-terminal to the insertion sequence may either be delivered to the ER or remain in the cytosol. In the former event, however, it is very unlikely that signal peptidase would cleave the pepide. Together, these findings favor the second scenario listed in the previous paragraph.

In addition to its interest as a basic problem in cell biology, understanding the mechanism of presentation of ES-peptides is of practical importance because we recently found that VV-ES $NP_{147-155}$ elicits better $NP_{147-155}$-specific T_{CD8+}-responses in H-2^d mice than VV-NP (N. Restifo, et al., unpublished observations). Further improvements in immunogenicity might be forthcoming from determining the site of cleavage of the peptide from the leader sequence by signal peptidase. Although the sequence at the leader-peptide junction is favorable for cleavage by signal peptidase, it is possible that most cleavage occurs at another site. In this case, antigenicity and possibly immunogenicity as well would almost certainly be enhanced by modifying the leader sequence such that cleavage exclusively occurred at the correct position.

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Peptide-Dependent Expression of HLA-B7 on Antigen Processing-Deficient T2 Cells[1]

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Class I MHC Ag presentation and cell surface expression largely depend on peptide transport into the ER/*cis*-Golgi by TAP, the transporter associated with Ag processing. Despite this dependency, class I MHC molecules are expressed at low levels on the surface of TAP-deficient T2 cells. We studied the peptide dependency of HLA-B7 expression in transfected T2 cells. HLA-B7 expression was affected by mutations at 19 out of 23 peptide-binding groove residues, but not by nine mutations outside of the peptide-binding groove. T2 cell surface HLA-A2, -B7, and -B51 had similar stabilities, and approximately half of these class I molecules had a long $t_{1/2}$ consistent with tight peptide binding. Using metabolically labeled T2 cells, HLA-A2-bound peptide eluted as five prominent peaks, but HLA-B7-bound peptide was not detected. In contrast, HLA-B7-eluted peptides were detected spectrophotometrically. These data suggest that HLA-A2 and HLA-B7 molecules utilize distinct TAP-independent peptide supply mechanisms to different degrees. Equivalent amounts of HLA-B7 from TAP$^-$ and TAP$^+$ cells yielded similar amounts of peptide, which had the characteristic HLA-B7 peptide-binding motif. The dependency of HLA-B7 cell surface expression on peptide-binding groove residues, the stability of cell surface class I molecules, and the ability to detect HLA-B7-bound peptide indicate that the low level expression on T2 cells is largely peptide dependent. TAP-independent peptide Ag presentation may allow immune recognition of intracellular pathogens that interfere with TAP-dependent peptide transport. *The Journal of Immunology*, 1996, 156: 3755–3764.

MHC molecules present peptide Ags to T cells. The supply of peptide Ags to class I MHC molecules is largely dependent on TAP,[3] the transporter associated with Ag processing, a heterodimeric ATP-binding cassette-transporter encoded by the *TAP1* and *TAP2* genes. T2 and 721.174 cells have a large deletion in the class II MHC region, which encompasses the *TAP1* and *TAP2* genes, and RMA-S cells have a defect in the *TAP2* gene (1–5). In the absence of a functional TAP heterodimer, most class I MHC molecules do not form stable heavy chain/β_2m complexes or egress from the ER/*cis*-Golgi. TAP-deficient cells express low levels of class I MHC molecules that are unstable at 37°C, but can be stabilized by reducing the temperature or adding exogenous peptides, mAbs, or β_2m (6–10). This suggests that surface class I molecules on TAP$^-$ cells do not contain tightly bound peptides.

T2 and RMA-S cells usually do not present peptide Ags derived from endogenous proteins (6, 8, 11), consistent with an essential role for TAP (5, 12–14). On T2 cells, HLA-A2 is expressed at low levels and binds peptides derived from signal sequences (15, 16), revealing a specialized TAP-independent peptide supply mechanism. Other class I MHC molecules expressed in T2 cells have no detectable peptide when measured by metabolic labeling with ^{3}H-labeled amino acids (10, 15). However, RMA-S and T2 cells present several antigenic peptides to CTL, albeit usually less efficiently than in TAP$^+$ cells (17–22). In addition, peptides recognized by alloreactive CTL can be isolated from RMA-S and T2 cells (23–25) and (Y. Shi, manuscript in preparation). Many of these antigenic peptides are not derived from signal sequences, suggesting that at least two TAP-independent peptide presentation pathways exist. Regurgitation of peptide Ags to the cell surface (26, 27), processing of proteins in the ER (22, 28), and ATP-independent, TAP-independent peptide transport into the ER (29, 30) may provide alternative sources of peptide for class I MHC molecules. The relative importance of these TAP-independent peptide presentation pathways or of peptide-independent class I MHC expression in TAP$^-$ cells is not clear; nor is it clear that all class I MHC molecules proportionally accrue peptides from the various alternative pathways.

In this report, we examine the hypothesis that class I MHC expression is largely peptide dependent, even in Ag-processing-deficient T2 cells. We compared the peptides bound by HLA-A2 and HLA-B7 molecules in T2 cells to test the hypothesis that different class I MHC molecules preferentially use distinct TAP-independent peptide supply pathways.

Material and Methods

Cell lines and mAbs

The following cells were generously provided: T2 (P. Cresswell, Yale University, New Haven, CT), 721.221 (referred to as .221; R. DeMars, University of Wisconsin, Madison, WI), and JY (J. Strominger, Harvard University, Cambridge, MA). T2 and .221 cells were transfected as described (31) with HLA-A2 (A*0201), HLA-A3 (A*0301), HLA-Cw3 (Cw*0301), HLA-B7 (B*0702), and HLA-B27 (B*2702 and B*2705) class I alleles or with HLA-B7 variant genes in the pHeBo vector. The HLA-B7 variants are designated by the single-letter amino acid code. For example, T2/S97R signifies T2 cells transfected with an HLA-B7 variant encoding a residue 97 Ser→Arg replacement. The following hybridomas were obtained from the American Type Culture Collection (Rockville,

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[3] Abbreviations used in this paper: TAP, transporter associated with Ag processing; β_2m, β_2-microglobulin; BFA, brefeldin A; ER, endoplasmic reticulum; MCF, mean channel fluorescence; mIgG, mouse IgG.

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MD): BB7.2, MA2.1, PA2.1, MB40.3, BB7.1, MB40.2, ME1, SFR8-B6, and BB7.6. The CR11-351 hybridoma was provided by S. Ferrone (New York University Medical Center, Valhalla, NY). The 22E1 and 2A1 hybridomas were provided by U. Hämmerling and 4B ascites was provided by S. Y. Yang (both of the Memorial Sloan-Kettering Cancer Center, New York, NY). The mAbs produced in serum-free culture were precipitated with ammonium sulfate and dialyzed. All mAbs were used at saturating concentrations.

Flow cytometry

Flow cytometry was done as previously described (31). The mean channel fluorescence data collected on 10,000 events with logarithmic amplification were converted to linear values using the following equation: Linear MCF = $10^{(\text{logarithmic MCF}/64)}$. To compare Ab binding by HLA-B7 variants to parental HLA-B7, the linear MCF for the HLA-B7 variant was divided by the parental HLA-B7 value. For two-color flow cytometry mAb ME1 was FITC conjugated, and mAbs BB7.2 and 2A1 were biotinylated according to standard protocols. The biotinylated mAbs were detected with phycoerythrin-strepavidin (Fischer, Pittsburgh, PA).

Cell surface stability of class I MHC molecules on T2 cells

T2/B7 cells were incubated in the presence or absence of BFA (1 μg/ml) for 0, 2, 4, 8, and 24 h. This concentration of BFA completely inhibits the cell surface expression of newly synthesized class I MHC molecules on T2/B7 cells (see Fig. 6, and data not shown). The cell surface expression of individual class I MHC molecules was measured by flow cytometry as indicated above, using mAbs BB7.2 (HLA-A2), ME1 (HLA-B7), and 2A1 (HLA-B51). To calculate the $t_{1/2}$ of the class I MHC molecules the following equation was used: log (linear MCF) = $-kt$ + C; and $t_{1/2} = (\ln 2)/k$, where k = the pseudo-first order rate constant for dissociation. The $t_{1/2}$ of the short-lived and long-lived complexes was calculated using the data points from 0 to 4 h, and from 4 to 24 h, respectively, using least squares linear regression analysis. Equivalent $t_{1/2}$ for short-lived and long-lived HLA-A2, HLA-B7, and HLA-B51 were determined in two independent experiments.

Metabolic labeling, affinity purification of class I MHC molecules, and HPLC separation of acid-eluted peptides

T2/B7 or T2/S97R cells (1×10^9) were labeled for 12 h with 5 mCi [^{3}H]Leu and 5 mCi [^{3}H]Pro (Amersham, Arlington Heights, IL) in Leu- and Pro-deficient RPMI 1640 medium (Life Technologies, Grand Island, NY) with 10% dialyzed FCS (Life Technologies). Cells were washed and lysed as described (15). Cleared cell lysates were applied to 1-ml columns of glycine-coupled, BB7.2-coupled and BB7-6-coupled Sepharose CL4B (Sigma, St. Louis, MO) connected in series. Columns were washed extensively and eluted with 50 mM diethylamine, pH 11.5, as described (15). The eluent was immediately neutralized with one-fifth volume 1 M Tris, pH 6.8, and concentrated with a Centricon-10 unit (Amicon, Beverly, MA). The concentrate was washed twice with 1 ml of 50 mM ammonium acetate, pH 7.5, and then acid denatured with 1 ml of 0.5% trifluoroacetic acid. Samples from the column eluates and the acid filtrates were mixed with scintillation fluid (Scintiverse II, Fischer) and counted (LS7800, Beckman, San Ramon, CA) to determine total HLA class I and the <10 kDa radioactivity. The Centricon-10 ultrafiltrate from acid treated HLA class I molecules was separated by reverse phase HPLC (2.1 × 250 mm C_{18} column, VyDac; Beckman 110B pump) as described (15). The eluent stream was mixed with three equivalents of scintillation fluid (Scintiverse LC, Fischer) and 6-s windows were directly analyzed with a radiochromatography detector (Radiomatic FLO-ONE\Beta, Packard, Downers Grove, IL). A second extraction (10% acetic acid and boiling) of the acid-treated HLA class I molecules failed to release additional peptides.

Affinity purification of class I MHC molecules; HPLC separation and sequencing of acid-eluted peptides

For sequencing HLA-B7-bound peptides, a slightly different protocol was used. HLA-B7 was affinity purified on a MB40.3-coupled Sepharose CL4B column (3-ml bed volume) from 6×10^9 JY or T2/B7 cells as described (32). Fractions containing HLA-B7 were identified by electrophoresing 1% of the fraction on a 15% SDS-PAGE mini-gel (MiniPROTEAN II, Bio-Rad, Hercules, CA) using the protocol of Laemmli (33) and staining with Coumassie brilliant blue (Kodak, Rochester, NY). HLA-B7 containing fractions were pooled and concentrated using Centricon-10 units. The retained HLA-B7 was washed twice with 1 ml of 50 mM ammonium acetate (EM Science, Gibbstown, NJ), pH 7.5. HLA-B7-bound peptides were eluted by two rounds of a 15-min 37°C incubation in 0.5 ml of 0.5% trifluoroacetic acid followed by filtration through a prerinsed Centricon-10 unit. The filtrates were pooled and stored at −80°C. The peptides were separated by reverse-phase HPLC (2.1- × 250-mm C_{18} column (VyDac, Hesperia, CA)) using an increasing acetonitrile gradient. Fractions eluting between 30 and 80 min contained nearly all of the 214 nm absorbing material and were, therefore, pooled for amino acid sequencing. Pooled peptides were sequenced by Dr. Alan Bergold in the University of Iowa College of Medicine Protein Structure Facility (Iowa City, IA), using an Applied Biosystems (Foster City, CA) model 470A protein sequencer and model 120A PTH analyzer.

Table I. *Expression of HLA class I molecules on .221 cells and T2 cells*

Cell Line	Ab Binding[a] mIgG	BB7.2	GAPA3	ME1	SFR8-B6	22E1
.221/pHeBo[b]	2	3	4	3	5	1
.221/A2	2	1528	4	2	5	2
.221/A3	2	10	815	2	3	2
.221/B7	2	11	2	1240	703	2
.221/B*2702	1	2	2	1427	2	2079
.221/B*2705	2	20	4	1597	4	1463
.221/CW3	2	12	6	20	60	2
T2	2	167	4	4	6	31
T2/A3	1	147	23	2	4	23
T2/B7	1	152	8	67	34	24
T2/B*2702	1	179	3	22	4	38
T2/B*2705	1	166	3	48	5	63
T2/CW3	1	191	5	4	11	20

[a] The average linear MCF from four experiments. The BB7.2 mAb detects HLA-A2; the GAPA3 mAb detects HLA-A3; the ME1 mAb detects HLA-B7; -B*2702, and -B*2705; the SFR8-B6 mAb detects the Bw6$^+$ HLA-B7, -Cw1, and -Cw3 (34); the 22E1 detects the Bw4$^+$ HLA-B*2702, B*2705, and B51; and mIgG is the mouse IgG-negative control.

[b] HLA-A-, -B-, -C-negative .221 cells transfected with the pHeBo vector alone.

Results

T2 cells express high levels of surface HLA-A2 relative to most other class I MHC molecules

Surface HLA-A2 is detected on T2 cells at about 10% the level on TAP-expressing .221 cells transfected with HLA-A2 (Table I). Transfected HLA-A3, B7, B*2702 and B*2705 are readily detected on T2 cells at about 2 to 6% the level on TAP-expressing .221 cells (Table I). The endogenous HLA-Cw1 and transfected HLA-Cw3 are barely detectable on T2 cells (Table I). Thus, other HLA-A and HLA-B molecules are expressed on T2 cells at about one-half to one-sixth the level of endogenous HLA-A2. Five conformation-dependent mAbs that recognize distinct HLA-A2 epitopes bind equivalently to T2 cells (Table II*A*). This suggests that HLA-A2 conformation is similar on T2 cells and TAP-expressing .221/A2 cells, consistent with the observation that T2 cell HLA-A2 contains bound peptide (15, 16). Four mAbs equivalently bind HLA-B7 expressed on T2 cells (Table II*B*). However, SFR8-B6 mAb binding may be slightly reduced and BB7.1 mAb binding is reduced threefold relative to other mAbs (Table II*B*). BB7.1 selectively binds HLA-B7 molecules loaded with different synthetic peptides.[4] Therefore, reduced BB7.1 binding may reflect unique HLA-B7-bound peptides or "empty" HLA-B7 on T2 cells.

Mutations in the peptide-binding groove selectively alter the expression of HLA-B7 on T2 cells

To test the influence of peptide-binding groove residues on T2 cell surface expression, we examined mAb binding to HLA-B7 variants with substitutions at residues predicted to interact with peptide, TCR, or both (35). All 33 variants are expressed at high levels

[4] Smith, K. D., B. E. Mace, A. Valenzuela, J. L. Vigna, J. A. McCutcheon, J. A. Barbosa, E. Huczko, V. H. Engelhard, and C. T. Lutz. Probing HLA-B7 conformational shifts by peptide-binding groove mutations and bound peptide with anti-HLA monoclonal antibodies. *Submitted for publication.*

Table II. *Detection of HLA-A2 and HLA-B7 on TAP⁺ .221 cells and TAP⁻ T2 cells*

A. Equivalent mAb detection of HLA-A2 expressed on .221 cells and T2 cells

		HLA-A2 Ab binding[a]				
Cell Line	mIgG	BB7.2	MA2.1	PA2.1	CR11-351	4B
.221/A2	2	1765	2038	2425	2258	2178
T2	2	154	174	167	192	227
		Expression of HLA-A2 on T2 relative to .221[b]				
		BB7.2	MA2.1	PA2.1	CR11-351	4B
Average		9%	9%	7%	9%	10%
SD		4%	3%	2%	3%	4%

B. Reduced BB7.1 binding to HLA-B7 expressed on T2 cells relative to .221 cells

		HLA-B7 Ab binding[a]					
	mIgG	MB40.3	BB7.1	MB40.2	ME1	SFR8-B6	BB7.6
.221/B7	2	1672	1657	1461	1658	1326	1484
T2/B7	1	100	23	68	88	43	70
		Expression of HLA-B7 on T2/B7 cells relative to .221/B7 cells[b]					
		MB40.3	BB7.1	MB40.2	ME1	SFR8-B6	BB7.6
Average		6.0%	1.4%	4.6%	5.3%	3.2%	4.7%
SD		1.2%	0.4%	1.1%	1.0%	0.6%	0.9%

[a] The average linear MCF from four experiments. Binding of mAbs to untransfected .221 cells, or untransfected T2 cells for the HLA-B7-binding mAbs, was equal to the mouse IgG (mIgG) control.

[b] The average and SD from four experiments of mAb binding to T2 or T2/B7 cells divided by the average mAb binding to transfected .221 cells.

on .221 cells, comparable to parental HLA-B7 (36, 37, and see footnote 4). Mutations at nine residues predicted to point toward the TCR, but not bound peptide, do not alter expression on T2 cells (Figs. 1 and 2). In contrast, the peptide-binding groove mutations dramatically alter expression on T2 cells (Figs. 1 and 2). Thirteen variants are barely detectable, four are detected at intermediate levels, four are detected at levels comparable to the parental HLA-B7, and two are detected at elevated levels (Fig. 1). This indicates that T2 cell surface expression is altered by peptide-binding groove mutations specifically and not HLA-B7 mutations in general. The range of HLA-B7 peptide-binding groove variant expression is comparable to the range of expression detected for individual *HLA-A*, *-B*, and *-C* alleles (Table I). This suggests that cell surface expression of class I MHC molecules on T2 cells is a function of the peptide-binding groove.

The E45A and S97R peptide-binding groove mutations, respectively, decrease and increase BB7.1 binding relative to the other mAbs (Fig. 1). Because BB7.1 binding is sensitive to specific HLA-B7-bound peptide (see footnote 4), BB7.1 may detect differences in peptides bound to the E45A and S97R variants in T2 cells. All Abs bind comparably to the remainder of the HLA-B7 variants except when specific epitopes are eliminated by the mutation (Fig. 1). To control for potential variation in individual transfectants, we also measured the expression of endogenous HLA-A2 and HLA-B51 (Fig. 1). With the exception of the S97R and the E45A variants, all transfectants express similar levels of HLA-A2 and HLA-B51. This indicates that changes in overall HLA class I surface expression on T2 cells cannot account for the range of HLA-B7 peptide-binding groove variant expression. The profound effect of several peptide-binding groove mutations likely indicates that HLA-B7 expression on T2 cells requires specific peptide binding. Alternatively, it is possible that "empty" HLA molecules are stabilized by particular peptide-binding groove residues.

T2 cells transfected with the HLA-B7 S97R variant showed a range of HLA-A2 and HLA-B51 expression (Fig. 3), averaging twofold lower HLA-A2 and fourfold lower HLA-B51 than untransfected T2 cells (Fig. 1). Similar HLA-A2 and HLA-B51 expression levels were observed in an independent pool of S97R-transfected T2 cells (data not shown). To demonstrate that reduced HLA-A2 and HLA-B51 expression was due to the presence of the S97R variant, we cultured cells in the absence of hygromycin B to permit loss of recombinant pHeBo episomes carrying the *HLA-B7* gene. After 15 days, approximately half of the cells had lost episomes, as evidenced by the loss of HLA-B7 expression (Fig. 3). Loss of the S97R episome resulted in a concomitant increase in HLA-A2 and HLA-B51 expression (Fig. 3). Further culture led to the complete loss of HLA-B7 expression and fully restored HLA-A2 and -B51 expression (data not shown). Loss of the parental HLA-B7 episome (Fig. 3), or episomes carrying E45A or six other HLA-B7 variants, did not alter HLA-A2 and HLA-B51 expression (data not shown). Thus, the S97R episome or the S97R class I molecules specifically inhibit cell surface expression of endogenous HLA-A2 and HLA-B51. Low HLA-A2 expression in T2/S97R cells also correlates with a reduced amount of immature HLA-A2 molecules immunoprecipitable by conformation-sensitive mAbs.[5] This suggests that the S97R HLA-B7 variant competes with HLA-A2 and HLA-B51 for factors required to assemble or stabilize heavy chain/β_2m complexes. β_2m probably is not a

[5] Vigna, J. L., K. D. Smith, and C. T. Lutz. Invariant chain associates preferentially with HLA class I/β_2-microglobulin heterodimers in an allele-specific manner, and association is influenced by peptide binding groove residues. *Submitted for publication.*

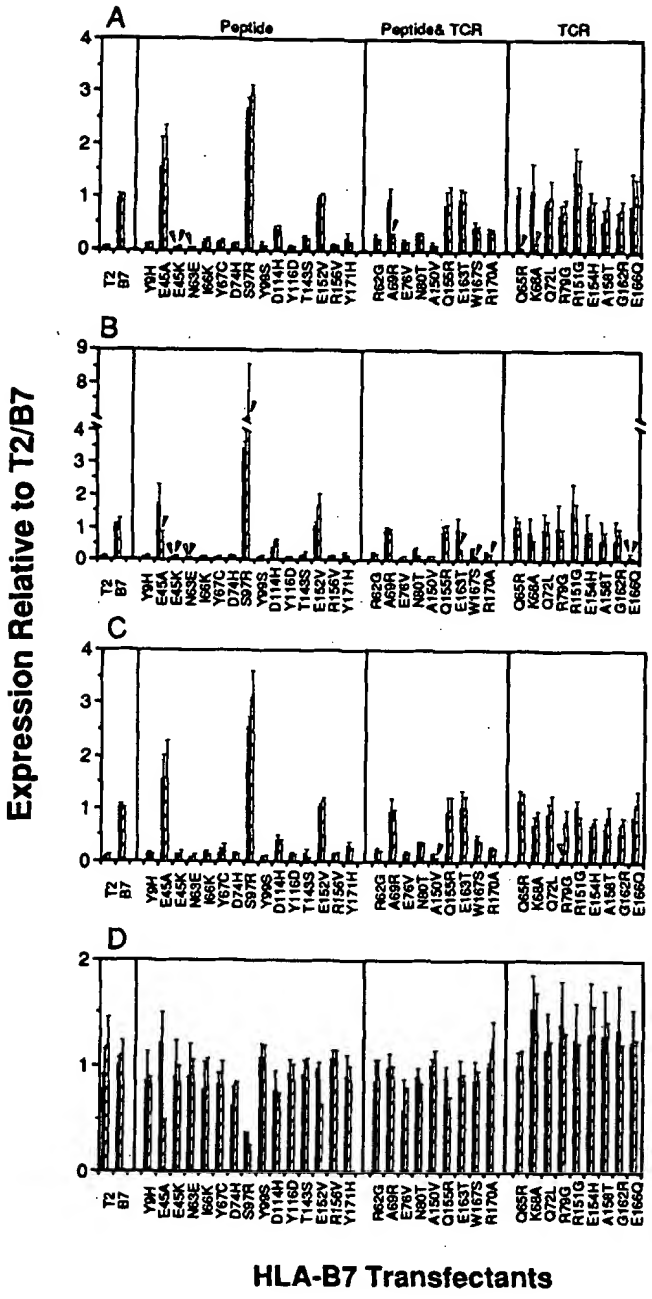

FIGURE 1. Mutations in the peptide-binding groove influence the cell surface expression of HLA-B7 on T2 cells. Shown on the *x*-axis are negative control untransfected T2 cells (T2), positive control HLA-B7-transfected T2 cells (B7), and T2 cells transfected with HLA-B7 variants divided into three categories based on side chain orientation (35). Shown on the *y*-axis are the mean values and one SD (error bars) from three to six experiments. Independent pools of transfectants gave identical results. *A*, Binding of anti-HLA-B7 mAbs MB40.3 (solid) and ME1 (hatched). *B*, Binding of anti-HLA-B7 mAbs MB40.2 (solid) and BB7.1 (hatched). *C*, Binding of anti-HLA-B7 mAbs SFR8-B6 (solid) and BB7.6 (hatched). *D*, Binding of anti-HLA-A2 mAb BB7.2 (solid) and anti-HLA-B51 mAb 22E1 (hatched). Mutations that affect mAb binding when expressed on .221 cells are marked with an open arrowhead. Additional mutations that affect specific mAb binding only when expressed in T2 cells are designated with a closed arrowhead. In general, these mutations are adjacent to other mutations that reduce mAb binding when expressed on .221 cells. For example, A69R reduces ME1 binding and is adjacent to residues 65 and 68 that are critical for ME1 binding (see footnote 4). Similar observations were made for E163T and E166Q.

limiting factor, because TAP transfection of T2 cells restores high HLA-A2 and HLA-B51 cell surface expression (38).

T2 cells express two populations of surface HLA class I molecules

Class I MHC molecules expressed on Ag-processing deficient T2 cells and RMA-S cells are less stable and have a shorter $t_{1/2}$ than class I MHC molecules expressed on TAP$^+$ cells (7, 10). This is consistent with the class I MHC molecules being empty (7, 10, 15) or binding long peptides that have fast off-rates (39). To measure HLA stability, we treated T2/B7 cells with BFA to block export of newly synthesized class I molecules to the cell surface and measured mAb binding at various times of incubation at 37°C (Fig. 4). HLA-A2, HLA-B7, and HLA-B51 show biphasic curves with rapid loss of mAb binding in the first 4 h, and a gradual loss of mAb binding thereafter. The biphasic curves indicate that there are at least two populations of surface class I MHC molecules on T2/B7 cells. The short-lived populations represent 36% of the HLA-A2, 56% of the HLA-B7, and 40% of the HLA-B51 molecules with $t_{1/2}$ of 4.8 h, 3.2 h, and 5.1 h, respectively. The $t_{1/2}$ of the long-lived populations is 17 h for HLA-A2, 29 h for HLA-B7, and 31 h for HLA-B51. The $t_{1/2}$ of the long-lived complexes is similar to those for other class I MHC molecules complexed to tight-binding peptides or on the cell surface of TAP$^+$ cells (40, 41), suggesting that the long-lived HLA molecules on T2 cells bind peptides with high affinity.

HLA-B7-bound metabolically labeled peptides are not detected on T2 cells

The presence of stable HLA-A2, -B7, and -B51 molecules on T2 cells suggested tight peptide binding. However, MHC-bound peptide has been detected only for HLA-A2 on T2 cells, with most of these peptides derived from signal sequences (15, 16). Peptides were not detected bound to H-2K^b, H-2D^p, HLA-A3 and HLA-B51 molecules expressed on T2 cells (15). To re-examine this question we metabolically labeled T2/B7 and T2/S97R cells with [^{3}H]Leu and [^{3}H]Pro for 12 h, affinity-purified HLA-A2 and HLA-B7, and acid-eluted HLA-bound peptides. Separation of acid-eluted peptides from HLA-A2 revealed five major peaks from both T2/B7 and T2/S97R cells (Fig. 5). Less HLA-A2 and less HLA-A2-eluted peptide were recovered from T2/S97R cells than T2/B7 cells (Table III), reflecting lower cell surface expression (Fig. 3). However, the recovery of HLA-A2-eluted peptide relative to HLA-A2 (Table III) and the relative abundance of each HPLC peak are similar for the two cell lines. This indicates that HLA-A2 is binding the same peptides in both T2/B7 and T2/S97R cells, and that S97R and HLA-A2 MHC molecules probably do not compete for the same peptides. No labeled peptide is detected for HLA-B7 purified from T2/B7 and T2/S97R cells (Fig. 5 and Table III), even though the S97R variant and HLA-A2 are expressed at equivalent levels. The absence of detectable peptides could indicate that HLA-B7 molecules are empty in transfected T2 cells, as proposed for other murine and human MHC molecules (10, 15). However, this is inconsistent with the similar stabilities of HLA-A2, HLA-B7, and HLA-B51 molecules on the T2 cell surface (Fig. 4). Cell surface HLA-B7 and B51 may be stabilized by serum-derived peptides, explaining their prolonged half lives and the failure to detect metabolically labeled peptide. To test this hypothesis, T2/B7 cells were acid stripped and incubated with or without serum (Fig. 6). BFA completely blocks the re-expression of class I MHC molecules on acid-stripped cells, but withholding serum does not inhibit cell surface recovery, indicating that serum derived peptides are not responsible for stable HLA-B7 and HLA-B51 expression on T2 cells.

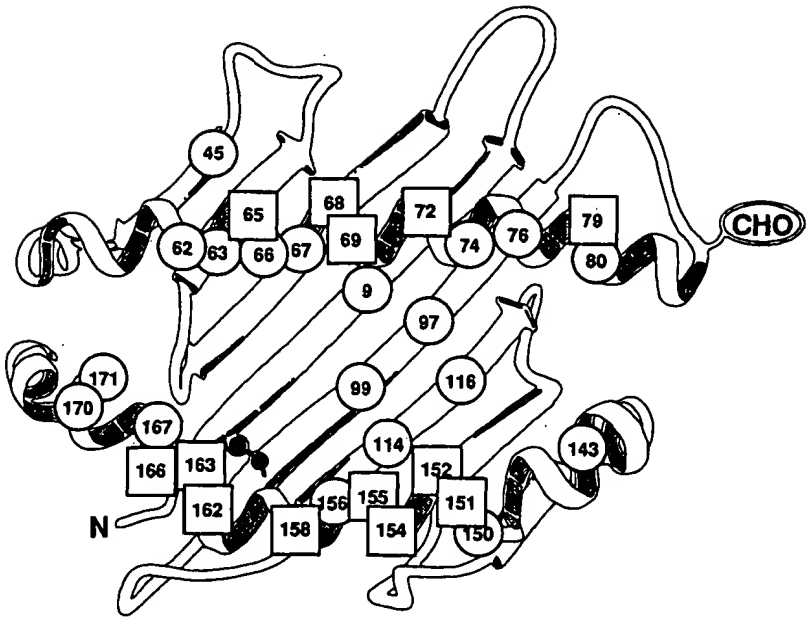

FIGURE 2. Mutations that alter the cell surface expression of HLA-B7 on T2 cluster in the peptide-binding groove. Mutated residues that decrease or increase HLA-B7 expression are shown as circles. Mutated residues that do not affect HLA-B7 cell surface expression are shown as squares.

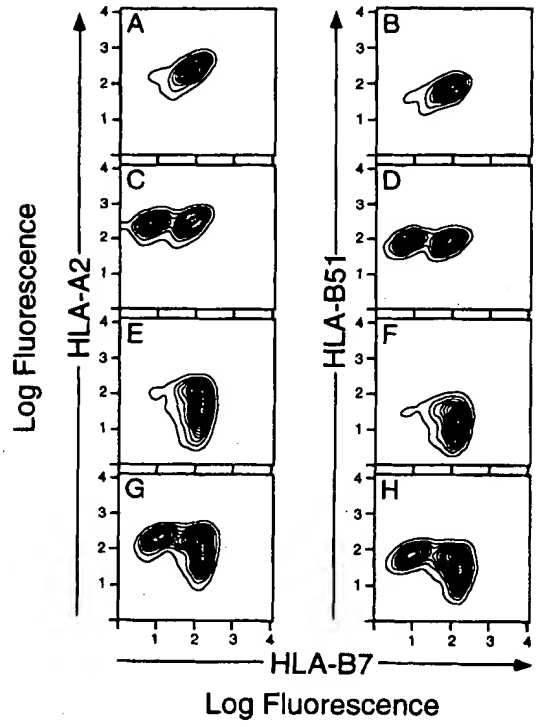

FIGURE 3. Reduced T2 cell surface expression of HLA-A2 and HLA-B51 requires the S97R episome. T2/B7 cells (*A*, *B*, *C*, and *D*) and T2/S97R cells (*E*, *F*, *G*, and *H*) were incubated for 15 days in the presence (*A*, *B*, *E*, and *F*) or absence (*C*, *D*, *G*, and *H*) hygromycin B. Cells were stained with anti-HLA-B7 mAb ME1, and anti-HLA-A2 mAb BB7.2 (*A*, *C*, *E*, and *G*), or anti-HLA-B51 mAb 2A1 (*B*, *D*, *F*, and *H*). Shown are fluorescence units on a four-decade log scale.

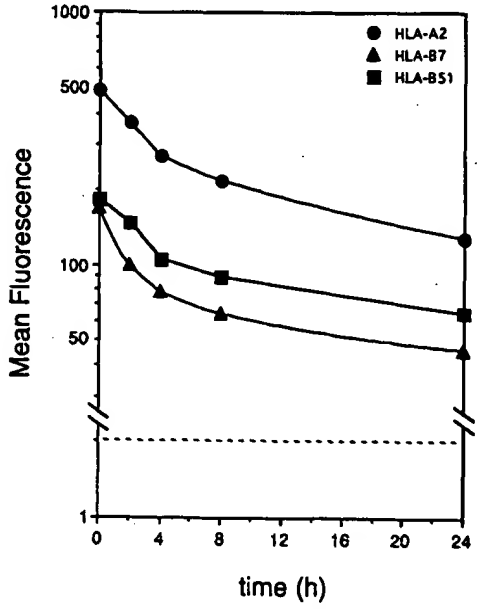

FIGURE 4. Two populations of HLA class I molecules are present on T2 cells. Surface expression of individual HLA class I molecules on BFA-treated (1 μg/ml) T2/B7 cells was measured with mAbs BB7.2 (HLA-A2), ME1 (HLA-B7), and 2A1 (HLA-B51). The dotted line shows mean fluorescence for the mouse IgG control (25 μg/ml).

Direct detection of a complex mixture of peptides bound to HLA-B7 on T2 cells

The inability to detect metabolically labeled peptide bound to HLA-B7 on T2 cells may indicate that HLA-B7-bound peptides are not significantly labeled. Signal peptides bound by HLA-A2 are expected to be labeled, rapidly processed, and bound by HLA-A2, but peptides derived from alternative sources may follow slower processing routes in TAP-deficient T2 cells. We affinity purified HLA-B7 from TAP$^+$ JY and TAP$^-$ T2/B7 cells and spectrophotometrically measured eluted peptides (Fig. 7). HLA-B7-eluted peptides from both JY and T2/B7 cells yielded complex HPLC profiles, indicating that both samples contained a diverse mixture of peptides. Compared with JY cells, the bulk of HLA-B7-eluted peptides from T2/B7 cells migrated more slowly on reverse-phase HPLC (Fig. 7), consistent with more hydrophobic residues. From T2/B7 cells we recovered 81% less HLA-B7 (60 μg from T2/B7 cells and 310 μg from JY cells) and 80% less HLA-B7-eluted peptides (comparing the integrated areas under the peaks eluting between 30 and 80 min, corrected for background).

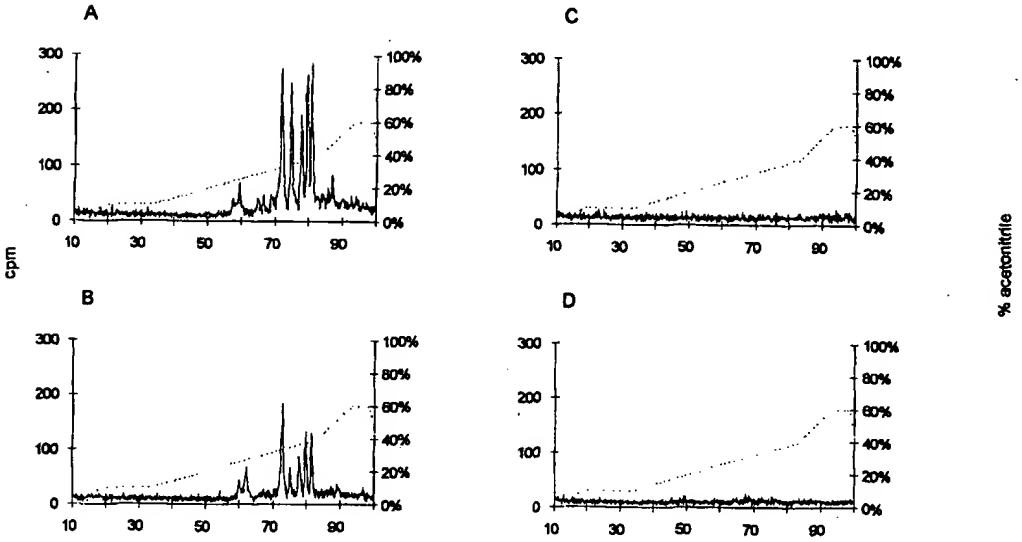

FIGURE 5. Metabolic labeling fails to identify peptides associated with HLA-B7 and the S97R variant expressed on T2 cells. The elution profile in cpm (6-s windows) for *A*) HLA-A2-eluted peptides from T2/B7 cells, *B*) HLA-A2-eluted peptides from T2/S97R cells, *C*) HLA-B7-eluted peptides from T2/B7 cells, and *D*) HLA-B7-eluted peptides from T2/S97R cells. The dotted line shows the acetonitrile gradient. Forty percent of the sample was analyzed in *A* and 80% of the sample was analyzed in *B*, *C*, and *D*.

Table III. *Summary of [^{3}H]Leu and [^{3}H]Pro counts for HLA class I purification and peptide elution*

Cell Line	Ag	cpm		
		Total	<10 kDa	% HLA[a]
T2/B7	—[b]	0.17×10^6	4,100	NA[c]
T2/B7	HLA-A2	3.46×10^6	82,980	2.4
T2/B7	HLA-B7	0.83×10^6	2,940	0.4
T2/S97R	—	0.21×10^6	1,260	NA
T2/S97R	HLA-A2	1.66×10^6	23,440	1.4
T2/S97R	HLA-B7	1.86×10^6	3,440	0.2

[a] The <10 kDa cpm divided by the total affinity-purified HLA cpm.
[b] Eluent from the glycine-coupled Sepharose column.
[c] NA, not applicable.

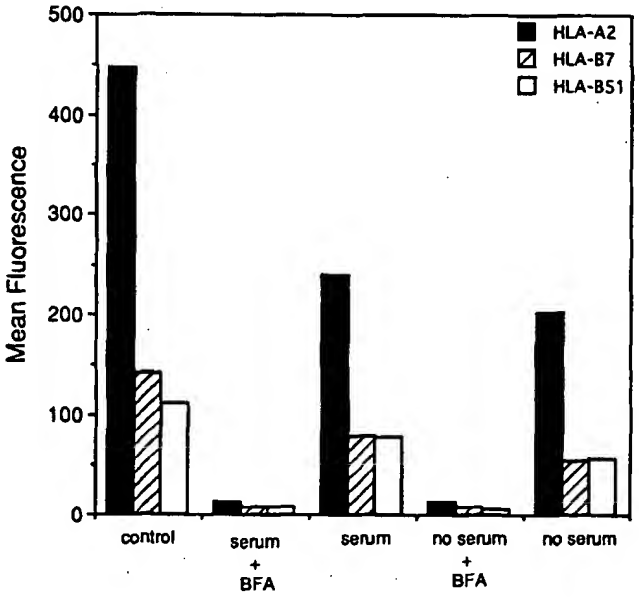

FIGURE 6. Regeneration of HLA class I molecules on T2 cells in the presence and absence of serum. The expression of individual HLA class I molecules on T2/B7 cells was measured with mAbs BB7.2 (HLA-A2), ME1 (HLA-B7), and 2A1 (HLA-B51). The first three data points (control) represent untreated T2/B7 cells; the remaining data points correspond to acid-stripped T2/B7 cells incubated at 37°C for 24 h in the presence or absence of serum and in the presence or absence of BFA (1 μg/ml), as indicated.

Therefore, roughly the same proportion of HLA-B7 molecules from TAP$^-$ T2/B7 cells and TAP$^+$ JY cells bind peptide.

To further investigate the HLA-B7-bound peptides from JY and T2/B7 cells, we sequenced pooled peptides by Edman degradation. HLA-B7-bound peptides from both cells had strong P2 Pro and P3 Arg signals (Table IV), typical of the HLA-B7 motif. The absolute amount of peptide recovered as determined by sequencing for HLA-B7-eluted peptides from T2/B7 cells was approximately threefold less than expected, based on the recovery for JY cells. This may in part be explained by a larger fractional loss of HLA-B7-eluted peptide from T2/B7 cells, since the 80% lower peptide concentrations and greater hydrophobicity led to greater fractional adsorption of dilute peptide during and after HPLC separation. In addition, the HLA-B7-eluted peptide from T2/B7 cells may on the average be longer than from JY cells. This is supported by the failure to detect any increase in the Leu signal in cycle 9 for T2/B7 peptide (Table IV). HLA-B7-eluted peptide from T2/B7 cells also had a less prominent Pro signal in cycle 2 and Arg in cycle 3, which decreased more gradually in subsequent cycles than HLA-B7-eluted peptide from JY. This suggests that some HLA-B7-eluted peptides from T2 cells may have N termini extending out of the peptide-binding groove or that some peptides use alternative anchors. HLA-B7-binding peptides with apparent N- and C-terminal extensions bind well to HLA-B7 in vitro (42).

Discussion

HLA-A and -B class I molecules are expressed at low, but easily detectable levels on T2 cells. The T2 cell surface expression of

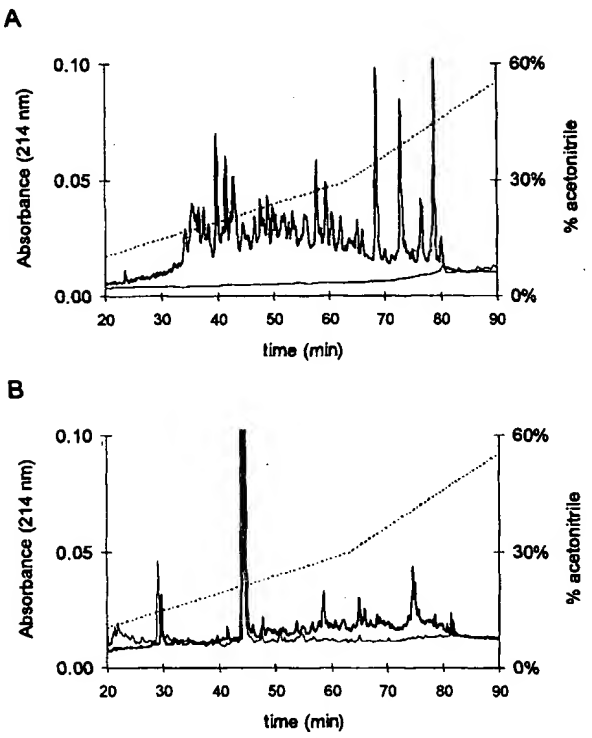

FIGURE 7. HLA-B7 eluted peptides from JY cells (*A*) and T2/B7 cells (*B*). The heavy line shows A_{214} for HLA-B7-eluted peptides. The thin line is the background control and shows A_{214} for the <10-kDa material that is acid eluted from nonspecific proteins purified on glycine-coupled columns. The dotted line shows the acetonitrile gradient. The large peak at 44 min in *B*, shown for both the HLA-B7 and control samples, represents an unidentified contaminant. This contaminant was most likely present in the peptide elution buffer or the Amicon filtration unit, since using new buffers and thoroughly rinsed filters eliminated the contaminant from the JY <10 kDa acid-eluted material.

HLA-B7 is influenced by peptide-binding groove residues. In addition, at least some HLA-B7 molecules expressed on T2 cells are occupied with peptides that have the characteristic HLA-B7 peptide binding motif. However, the HPLC profile and sequencing data indicate that HLA-B7-bound peptides in TAP$^-$ T2 cells are qualitatively different, possibly more hydrophobic and longer, than HLA-B7-bound peptides in TAP$^+$ JY cells. On T2 cells approximately half of the HLA-A2, -B7, and -B51 molecules have a long $t_{1/2}$, consistent with tight peptide binding. The short-lived complexes may represent loosely bound MHC/peptide complexes or empty molecules. Together these data support the hypothesis that even the low levels of class I MHC molecules on T2 cells largely require peptide binding for the significant egress from the ER and cell surface expression of properly folded molecules.

HLA-A2 binds peptides derived from signal sequences in both TAP$^-$ T2 cells and TAP$^+$ T1 cells (16). Several prominent HLA-B7-eluted peptides from TAP$^+$ JY cells also are derived from signal sequences (43). Therefore, we anticipated prominently labeled signal peptides eluted from HLA-B7 in T2/B7 cells. Because no labeled peptides were detected, HLA-B7-binding signal peptides may not be presented efficiently in T2 cells. This suggests that presentation of some signal peptides by conventional class I MHC molecules is TAP dependent. Presentation of a signal peptide by the class Ib MHC molecule, H-2Qa-1, also is TAP dependent (44). We compared the sequences of signal peptides that have been eluted from class I MHC molecules to determine if any structural relationships exist between TAP-dependent and TAP-independent signal peptides. Signal sequences can be divided into three regions (45). The N region is near the amino terminus and usually contains a net positive charge. The H region contains a hydrophobic core at least seven amino acids long. The C region contains the signal peptidase recognition site and is predicted to be accessible to ER lumen proteins. All of the peptides that are apparently presented in a TAP-dependent manner (15, 44, 46, 47) are derived from the N region (data not shown). These N region-derived peptides contain charged residues that are thought to anchor signal peptide N termini to the cytosolic face of the ER membrane (45). This suggests that charged N regions of cleaved signal peptides diffuse back into the cytosol and are processed by cytosolic proteases. In some instances these processed peptides are transported back into the ER via TAP.

Peptides derived from three signal sequences are efficiently presented by HLA-A2 molecules in T2 cells (15, 25, 46). IP-30 and SSRα donate peptides that are derived from the C terminus of the signal sequence (15, 25, 46). In contrast, the calreticulin signal sequence peptide is derived from the N region (25). However, the calreticulin signal peptide N region lacks charged residues and therefore may diffuse through the ER membrane into the lumen to bind HLA-A2. Diffusion of the hydrophobic N terminus through the ER membrane has been proposed for a truncated invariant chain (48). Deletion of the charged N-terminal cytosolic domains creates hydrophobic N terminus that appears to diffuse into the ER membrane, uncovering a cryptic signal peptidase cleavage site and creating a secreted form of invariant chain. To address possible diffusion of the calreticulin signal peptide N region into the ER membrane, we simulated the addition of 10 alanine residues to the calreticulin N terminus and calculated scores for potential signal peptidase cleavage sites, using AnalyzeSignalase version 2.03 software (49). The predicted diffusion of the calreticulin signal peptide into the ER membrane uncovers a second strong signal peptidase cleavage site, which generates the same 10 amino acid peptide presented by HLA-A2 (data not shown). In the calreticulin peptide/HLA-A2 co-crystals, the calreticulin peptide C-terminal glycine extends out of the peptide-binding groove, indicating that an optimal nine-amino acid peptide is not generated in T2 cells (50). Except for signal peptidase cleavage, processing of peptide C termini in the ER may be inefficient (51). This would limit HLA-binding signal sequence-derived peptides to those that have C termini at or near predicted signal peptidase cleavage sites or which require TAP transport after processing in the cytosol.

The HLA-A2-eluted peptide contained about 2% of the radioactivity associated with affinity-purified HLA-A2 molecules from T2/B7 and T2/S97R cells (Table III). This is similar to the HLA-A2 peptide fraction eluted from T2 cells and the TAP$^+$ parental T0 cells (15). The expected value based on Leu and Pro (or Leu and Lys for Wei and Cresswell (15)) content in HLA-A2 and HLA-A2-binding peptides (46) is 8 to 10%, assuming equal labeling of HLA-A2 and peptide. The difference between the observed and expected values suggests either that peptide disassociated from HLA-A2 during purification or that more peptide than HLA-A2 is unlabeled.

It is easier to detect labeled peptides if the radioactivity is concentrated in a few dominant peptide peaks than if the radioactivity is spread over many peptides. This may, in part, account for the absence of detectable HLA-B7-eluted peptides from T2 cells. However, unlabeled HLA-B7-eluted peptides were easily detected, indicating a lag between labeling of donor proteins and the subsequent generation, transport, and HLA binding of peptides. For most HLA-bound peptides this lag is anticipated since donor proteins with a variety of life spans supply peptides for class I MHC

Table IV. *Sequencing of pooled HLA-B7-eluted peptides from T2/B7 cells and JY cells*

Cycle	Amino Acid (pmol)[a]																		
	A	R	N	D	E	Q	G	H	I	L	K	M	F	P	S	T	W	Y	V
Sequencing of HLA-B7-Eluted Peptides from T2/B7 Cells[b]																			
1	27.8	7.2	8	0	3	0	21.6	1.3	8.2	9.4	1.2	0.9	2.5	4.6	13.1	2.9	0	2.4	4.9
2	15.8	6.7	2.7	0	3.6	0.4	17.5	1.1	9.1	8.4	0.3	0.7	1.6	20.7	5.7	1.3	0	2	5.6
3	12.9	14.8	2.3	2.4	3.4	2.7	11.5	1.5	6	5.5	0	0.9	1.3	12.7	3.1	0	0	2.6	4.5
4	11.3	10	1.9	1.8	3.9	1.9	11.3	1.5	4.8	5.1	0.1	0.9	1.5	11.8	3.2	0	0	2.3	3.9
5	8.7	8.7	0.5	0.8	3.8	1.5	11.8	1.3	5.2	4.9	0.1	0.8	1.5	10	2.1	0.2	0	1.9	4.1
6[c]																			
7[c]																			
8	9	8.8	1.3	1.1	4.4	2.1	15.2	1	4.8	4.7	0.2	1.8	1.9	12.2	d	0	0	4.1	5.1
9	4.3	5.4	d	0.8	2.1	0.5	4.3	0.9	1.2	2.9	0	0.5	1	4.3	1.9	0	0	1.8	2.1
10	2.8	3.9	d	0	1.7	0.5	3.5	0.5	0.7	2.3	0	0.4	0	3.6	0.4	0	0	1.2	1.7
Sequencing of HLA-B7-eluted peptides from JY cells[b]																			
1	516.6	572.0	39.0	23.5	70.6	51.3	217.2	20.9	47.4	94.4	142.7	24.4	20.1	15.7	198.6	73.2	2.7	49.6	66.6
2	173.5	42.1	5.1	61.4	14.9	5.6	42.2	3.6	16.8	15.3	16.3	2.7	1.1	787.2	22.0	9.5	1.4	4.9	90.4
3	109.6	625.0	23.5	38.3	22.6	36.2	33.2	16.7	12.1	19.9	37.7	41.3	9.5	170.8	69.1	15.7	1.4	16.6	15.7
4	128.2	223.7	38.4	55.0	81.7	39.5	86.6	20.7	15.2	47.9	84.8	10.8	12.6	137.2	51.3	29.9	2.7	20.9	27.5
5	90.7	172.5	35.3	35.9	86.3	41.0	77.8	16.4	23.9	29.4	48.3	6.5	8.8	141.2	47.4	26.1	1.3	13.9	53.8
6	50.0	336.2	22.2	24.1	30.8	24.0	73.8	14.4	21.9	26.4	30.2	7.1	15.0	74.1	35.4	50.9	1.3	14.5	37.1
7	49.7	187.8	24.9	20.7	33.3	28.0	46.3	12.5	20.5	39.7	22.2	10.0	6.8	53.3	47.5	33.7	1.4	14.2	38.8
8	59.1	89.7	19.2	13.5	44.3	28.2	47.7	7.0	11.2	32.0	15.5	9.9	5.0	20.9	33.7	32.8	0.4	10.5	36.0
9	33.1	55.1	8.4	7.6	22.6	17.1	20.8	4.4	8.5	103.6	8.5	11.3	9.0	9.4	18.3	17.8	0.1	6.3	47.7
10	13.3	25.0	3.6	3.6	8.8	6.9	8.2	2.3	3.2	40.2	3.9	4.4	3.9	4.6	14.9	7.7	0.0	2.9	14.6
11	6.8	13.2	2.0	2.3	4.0	2.4	4.3	1.5	1.7	25.3	2.2	2.4	1.7	2.9	5.1	3.2	0.0	1.4	6.6
12	3.1	8.2	1.0	0.3	2.0	1.0	2.9	0.0	0.9	10.9	0.7	1.6	0.8	2.3	1.3	1.9	0.0	0.9	3.8
13	2.9	5.4	0.9	0.5	1.7	0.8	2.7	0.6	0.3	5.7	1.2	0.7	0.5	1.7	1.8	1.4	0.0	0.6	2.3

[a] The signals that increase by greater than 100% over the previous cycle are underlined.
[b] The pooled sequencing data is from peptides eluted from 60 μg of HLA-B7 purified from 6×10^9 T2/B7 cells and 200 μg of HLA-B7 purified from 6×10^9 JY cells.
[c] The computer lost communication with the analyzer during cycles 6 and 7 losing the amino acid sequencing data.
[d] The amount of amino acid could not be quantified due to a co-eluting contaminant.

molecules. For many signal sequences the lag between labeling and generation of HLA-binding peptides is likely to be very short, since ^{3}H-labeled signal peptides are immediately transported into the ER. Unprotected ER peptides are rapidly exported (52–54), prohibiting HLA binding to long-lived free ER peptides. If HLA-A2 is bound entirely to signal peptides in T2 cells, the labeling of HLA-A2-bound peptides is expected to approximate 8 to 10%. Therefore, a fraction of the HLA-A2 may bind unlabeled peptide, similar to HLA-B7 in T2 cells. Using mass spectroscopy, approximately 40% of the T2 cell peptides eluted form HLA-A2 was accounted for by seven prominent peptides of which five have been identified as signal peptides (16, 46). The remaining HLA-A2 binding peptides were a complex mixture, perhaps representing peptides that are poorly labeled during 6- to 12-h pulses with ^{3}H-labeled amino acids.

Using metabolically labeled T2 cells, we detected peptides bound to HLA-A2, which migrate as five prominent HPLC peaks. In contrast, we did not detect labeled peptides bound to HLA-B7 or even to the S97R variant, which is expressed at levels equal to HLA-A2 on T2 cells. This and published data indicate that signal peptides are labeled, rapidly transported into the ER, processed, and bound to HLA-A2. In contrast, peptides bound by HLA-B7, and presumably other class I molecules expressed in T2 cells, traverse a kinetically slower route. Our data clearly support the hypothesis that HLA-A2 and HLA-B7 preferentially utilize different TAP-independent peptide supply pathways.

TAP$^-$ RMA-S cells present vesicular stomatitis virus, Sendai virus, and Rauscher murine leukemia virus peptides, albeit less efficiently than TAP$^+$ RMA cells (17, 18, 20, 55). T2 cells poorly present Ag when infected with natural pathogens (8, 11, 56). However, T2 cells transfected with minigenes driven by strong heterologous promoters or infected with recombinant vaccinia virus present some peptide Ags to CTL (21, 22). The decreased efficiency of Ag presentation and the lower levels of surface class I molecules in TAP-deficient RMA-S and T2 cells likely reflect both a slower and smaller supply of peptides. Thus, a major role of TAP is to rapidly transport peptide Ags for T cell surveillance early in infection. This enables T cells to fight intracellular pathogens with generation times measured in hours or minutes. The overwhelming dependence of class I Ag presentation on TAP probably reflects co-evolution of class I MHC and TAP. Residual peptide presentation in T2 cells may represent primitive pathways that have been superseded by the TAP pathway. Herpes simplex virus ICP47 interferes with TAP transport of peptides (57, 58), suggesting that TAP-independent pathways may be advantageous in certain circumstances.

TAP1-deficient mice have significantly reduced class I MHC expression and CD8$^+$ T cells (59), however the CD8$^+$ T cells that do emerge in the periphery respond to allogeneic cells and syngeneic TAP$^+$ cells (60, 61). It is not yet known whether *TAP1*-deficient mice mount relevant CD8$^+$ T cell responses to intracellular pathogens. A rare human immunodeficiency linked to mutations in the *TAP2* gene suggests that this may be possible (62). Two immunodeficient siblings suffer from persistent bacterial infections. The younger patient has a reduced CD8$^+$ T cell count,

but the older patient has a nearly normal $CD8^+$ T cell count, suggesting that a small number of peripheral T cells expanded in response to Ag (62). These data indicate that the class I MHC-dependent arm of the immune system may retain some function even in the absence of TAP.

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The Effect of the Proteasome Inhibitor Lactacystin on the Presentation of Transporter Associated with Antigen Processing (TAP)-Dependent and TAP-Independent Peptide Epitopes by Class I Molecules[1]

Ailin Bai and James Forman[2]

Cells were treated with two proteolytic inhibitors, *N*-acetyl-leucyl-leucyl-norleucinal and lactacystin, the latter reported to be a specific inhibitor for the proteasome. Both inhibitors retarded the maturation of endo-H-resistant forms of murine and human class I molecules from their endo-H-sensitive precursors in cell lines with functional TAP proteins. HLA-A2 maturation readily occurs in TAP-deficient T2 cells, and it has been shown that the peptides associated with A2 are derived from the leader segment of proteins in the secretory pathway. This maturation is inhibited by *N*-acetyl-leucyl-leucyl-norleucinal but not lactacystin, indicating that the proteasome is not required for the generation of HLA-A2 binding peptides in these cells. The murine class Ib molecule Qa-1^b presents a leader peptide derived from D-end class I molecules to alloreactive CTL. Since this presentation is dependent on the expression of TAP proteins, we determined if this requirement reflects a need for the proteasome to process this peptide. We found that lactacystin did not inhibit the maturation of endo-H-resistant forms of Qa-1^b that are dependent on this leader peptide for its maturation, nor did it inhibit the expression of this peptide-Qa-1^b complex in a functional assay. Thus, unlike conventional cytosolic peptides, leader peptides (regardless of whether they are dependent on TAP for their presentation) do not require the proteasome for processing. *The Journal of Immunology,* **1997, 159: 2139–2146.**

MHC class I molecules present peptide fragments derived from endogenously synthesized proteins for CD8$^+$ T lymphocyte recognition. The majority of antigenic peptides are generated in the cytosol and subsequently translocated by the TAP into the ER,[3] where they are loaded to the newly synthesized class I molecules (1). The proteolytic systems responsible for the generation of these peptides have not been well characterized. Certain peptides derived from leader sequences have been shown to be able to enter the class I Ag presentation pathway (2–5). Some of these leader peptides are produced in the ER and can be displayed on the cell surface in TAP-deficient cells (2, 3). Others are presented in a TAP-dependent manner (4, 5). Little is known about the processing of leader peptides. Peptide trimming in the ER has been described (6, 7). Elliott et al. (7) reported that extension of 40 amino acid residues can be trimmed from the N terminus of a known epitope in the ER. How ER proteases contribute to the generation of class I peptide pool is not clear. The generation of TAP-dependent leader peptides is even more elusive. One such peptide is termed Qdm (Qa-1 determinant modifier), which is derived from the leader sequences of certain murine D-end class I molecules and is presented by the class Ib molecule Qa-1^b (4). Unexpectedly, the presentation of this peptide is dependent on the functional TAP transporters. The processing mechanism of the Qdm peptide and the nature of the TAP dependence are not understood. One hypothesis is that the Ag processing capacity in the ER is limited for some peptides; the real epitope can only be generated in the cytosol.

The proteasome, a multicatalytic protease complex present in the cytosol and the nucleus, has been implicated in Ag processing (8). Two of the proteasome subunits (LMP2 and LMP7) are encoded in the MHC region and induced by IFN-γ (9). The presence of these subunits can modify the activity of the proteasome and affect class I Ag presentation (10, 11). However, whether most class I-presented peptides are generated by the proteasome has not been conclusively established. So far the most direct evidence comes from studies with peptide aldehyde inhibitors (12–14). Some of these inhibitors can inhibit class I Ag presentation, and their inhibitory capacities are correlated with their potencies to inhibit the purified proteasome in vitro. However, these inhibitors also act on other proteases. At least in one case, their nonproteasome directed inhibitory activities contribute to their inhibition of class I Ag processing (15).

Recently, lactacystin (a natural product isolated from streptomyces) has been shown to be a more specific proteasome inhibitor (16). Certain proteasome β subunits are the specific cellular targets of lactacystin. The amino-terminal threonine residues of these β subunits, which have been shown to be the catalytic active sites by the crystal structure and site-directed mutagenesis studies, are covalently modified by lactacystin (17, 18). Currently, no other proteases are known to be inhibited by lactacystin. In the present study, we take advantage of this specific proteasome inhibitor to investigate the involvement of the proteasome in class I Ag presentation of both TAP-dependent and -independent peptide epitopes.

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[3] Abbreviations used in this paper: ER, endoplasmic reticulum; LLnL, *N*-acetyl-leu-leu-norleucinal; Vac, vaccinia virus; HPV, human papilloma virus; endo-H, endo-β-*N*-acetylglucosaminidase H.

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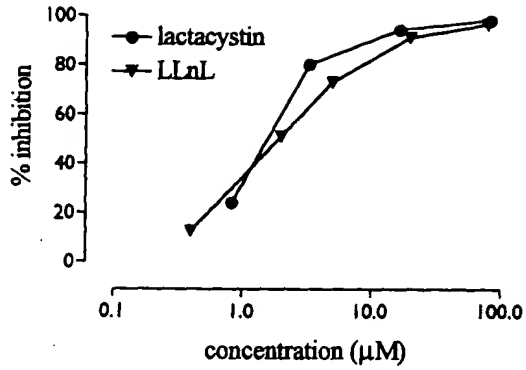


FIGURE 1. In vitro inhibition of the purified 20S proteasome by lactacystin (●) and LLnL (▼). One microgram of purified 20 S proteasome was incubated with 1 to 100 μM lactacystin in 6 μl of 50 mM Tris-HCl, pH 8.0, 5 mM DTT at 37°C for 20 min. The fluorescence assay was conducted in 1 ml of substrate buffer (50 mM Tris-HCl, pH 8.0, 1 mM DTT, 50 μM Suc-Leu-Leu-Val-Tyr-AMC) at 37°C. The velocities were calculated from the linear region of reaction progress curves (21). For LLnL, the proteasome and the inhibitor were added directly into the substrate buffer, and the final concentrations of LLnL in the reactions represented the effective concentrations.

Materials and Methods

Reagents

Lactacystin was purchased from Dr. E. J. Corey (Harvard University, Boston, MA) and was dissolved in water at 5 mM. *N*-acetyl-Leu-Leu-norleucinal (LLnL) was prepared as a 40-mM stock in DMSO. Brefeldin A (BFA, Sigma Chemical Co., St. Louis, MO) was dissolved in methanol at 10 mg/ml. The synthesis of the Qdm peptide was described previously (4).

Cell lines

RMA, RMAS, OVA-expressing EL4 transfectant E.G7-OVA (provided by Dr. Michael Bevan, University of Washington, Seattle, WA), and the generation of Qa-1^b-specific CTL clones 3C9 and 5D2 have been described previously (4, 19, 20). The Qa-1^b expressing stable L cell transfectant L-g37 was a kind gift of Dr. M. Soloski (Johns Hopkins University, Baltimore, MD). W12.1 was generated by the stable transfection of L cells with the L^d gene. T2 and C1R.A2 cell lines were kindly provided by Dr. P. Cresswell (Yale University). The OVA specific T cell hybridoma B3Z was a kind gift of Dr. N. Shastri (University of California, Berkeley). All cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS.

Antibodies

28-14-8s (HB27, anti-D^b or L^d), FD441.8 (TIB213, anti-LFA-1), and MA2.1 (HB54, anti-HLA-A2) were obtained from American Type Culture Collection (ATCC, Rockville, MD). 1G12 (anti-human transferrin receptor) was provided by Dr. P. Cresswell (Yale University, New Haven, CT). A rabbit anti-Qa-1^b serum raised against the C-terminal region of the protein was a generous gift of Dr. M. Soloski (Johns Hopkins University).

Pulse-chase and immunoprecipitation

Cells were starved in methionine-free medium for 90 min at 37°C, then pulse labeled with 0.2 mCi/ml Tran^{35}S label (ICN, Irvine, CA) for 20 min. Labeling medium was removed by centrifugation and cells were chased with complete medium containing excess methionine for various times. Both lactacystin and LLnL were tested in vitro for their ability to inhibit the hydrolysis of a fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC (Fig. 1). The purified 20S proteasome and the fluorogenic peptide were provided by Dr. G. DeMartino (University of Texas Southwestern Medical Center at Dallas). Approximately 80% inhibition was noted when the inhibitors were added in the low μM range, and close to 100% inhibition was seen at 20 μM. The inhibitors were titrated in vivo, and optimal inhibition concentrations were found to be 80 μM for lactacystin and 250 μM for LLnL (data not shown). These concentrations were therefore used in all of the experiments described below. When indicated, lactacystin or LLnL was included through the methionine starvation and pulse-chase process. 3×10^6 to 10×10^6 cells were lysed on ice with 0.5 ml of lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM iodoacetamide, 1 mM PMSF, 0.1 trypsin inhibitor U/ml aprotinin) for 30 min. The postnuclear supernatants were precleared overnight at 4°C with fixed *Staphylococcus aureus* cells (Sigma Chemical Co.) or protein-G Sepharose (Pierce, Rockford, IL). The target molecules were immunoprecipitated by the sequential incubations with Abs and protein-A or -G Sepharose beads (Pierce). The immunoprecipitates were washed three times and endo-H (Boehringer Mannheim, Indianapolis, IN) digestion was performed as described (22). Samples were separated by SDS-PAGE. The results were analyzed with a PhosphorImager (Molecular Dynamics, Seal Beach, CA). The quantitative data are presented either as the percent of endo-H-resistant molecular forms (activity in endo-H-resistant band/activity in endo-H-sensitive + endo-H-resistant bands × 100) or amount of total activity detected in the endo-H-resistant band.

Virus infection

Recombinant vaccinia virus containing the D^d gene (Vac-D^d) was kindly provided by Dr. J. W. Yewdell (NIAID, National Institutes of Health, Bethesda, MD). Vac-HPV has a human papilloma virus (*HPV*) gene recombined into the vaccinia genome. For immunoprecipitation, L-g37 cells were infected with wild-type or recombinant vaccinia viruses at a multiplicity of 50 for 2 h before methionine starvation. For the CTL assay, L-g37 cells were infected with viruses at a multiplicity of 10 in the presence or absence of the inhibitors for 3.5 h. BFA was added to a final concentration of 3 μg/ml 30 min before the end of infection.

CTL assay

The standard ^{51}Cr release was performed as described except that BFA was added at 1 μg/ml (4).

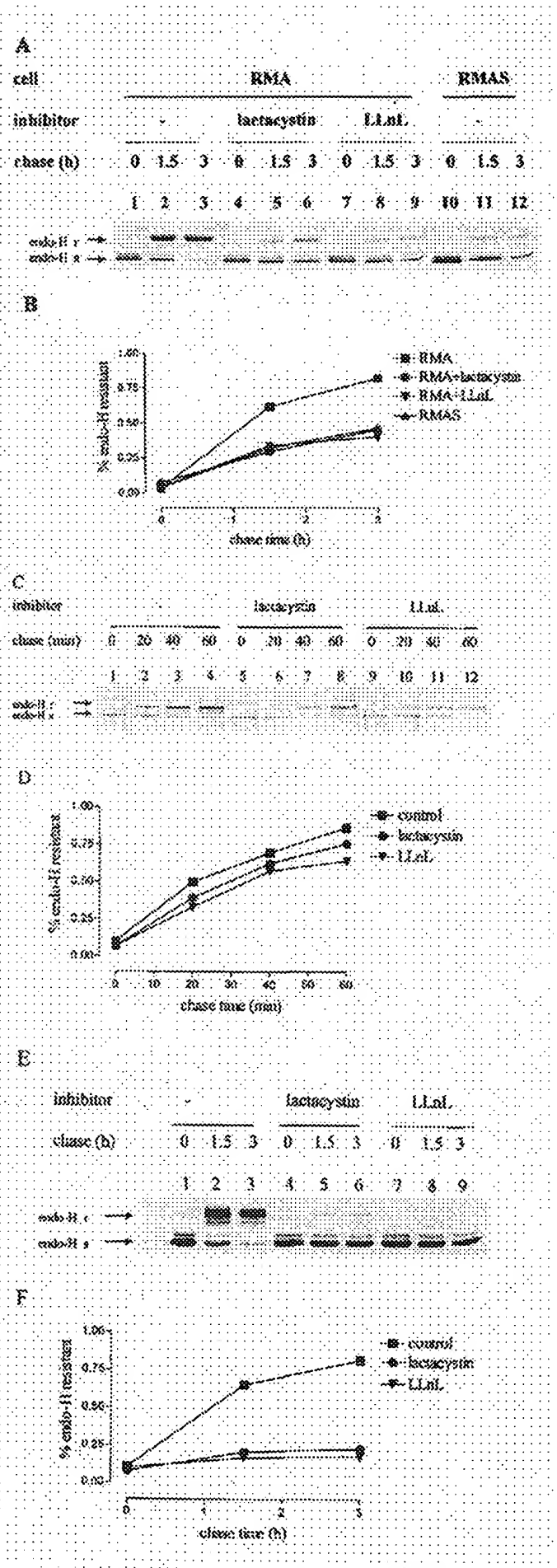
Acid wash/recovery and T cell activation assay

The newly generated $OVA_{257-264}/K^b$ on E.G7-OVA cells was measured as described (23). Pre-existing peptide/MHC class I complexes on the cell surface were removed by the exposure to mild acid solution (131 mM citric acid, 66 mM disodium phosphate, pH 3.1) at 25°C for 3 min. After neutralization in 30 vol of complete RPMI 1640 medium and washing in PBS, cells were either fixed with 1% paraformaldehyde immediately or resuspended in complete RPMI 1640 medium in the presence or absence of the inhibitors. At various times of recovery, cells were fixed and used as APC. To measure the surface expression of $OVA_{257-264}/K^b$, 5×10^4 E.G7-OVA cells were incubated with 5×10^4 B3Z T hybridoma cells at 37°C for 5 h. Upon activation, B3Z cells express LacZ activity that can be measured by a chromogenic reaction (24). Substrate solution containing 0.2 mM CPRG (Calbiochem, la Jolla, CA) and 0.5% Nonidet P-40 in PBS was added after the cultures were washed with PBS. The T cell response was represented by the absorbance at 575 nm.

Results

Lactacystin and LLnL inhibit the maturation of class I molecules D^b and L^d

Peptide ligands are essential for the stable assembly of class I molecules. Only those class I molecules that bind peptides can be efficiently released from the ER and transported to the Golgi where their *N*-linked oligosaccharides are processed into mature forms. A lack of peptides leads to the prolonged retention of class I molecules within the ER and therefore a decreased rate of maturation. Two inhibitors, lactacystin and a peptide aldehyde inhibitor LLnL, were tested for their effects on class I maturation with pulse-chase experiments. In control RMA cells, most D^b molecules are processed into mature forms after a 3-h chase (Fig. 2*A*, *lane 3*). In the presence of lactacystin or LLnL, only a small amount of D^b acquires endo-H resistance after the same period of chase (*lanes 6* and *9*). The maturation pattern of D^b in inhibitor-treated RMA cells resembles that in TAP-deficient RMAS cells (*lanes 10–12*), indicating that this may result from a decreased peptide supply. A PhosphorImager was used to quantitate the results, and the percent of endo-H-resistant material is shown in Figure 2*B*. To exclude the possibility that these inhibitors affect other processes in class I maturation rather than peptide supply, the maturation of LFA-1, which is independent of peptide supply, is examined in the presence of these inhibitors. As shown in Figure 2, *C* and *D*, lactacystin



does not inhibit the maturation of LFA-1. Although less material is recovered from LLnL treated cells, the kinetics of LFA-1 maturation is only slightly affected. We also tested these inhibitors in an L cell transfected line with a different class I molecule (Fig. 2, *E* and *F*). The maturation of L^d in this line is almost completely blocked by lactacystin or LLnL. These results suggest that both inhibitors block the maturation of class I molecules most likely by depriving them of an adequate peptide supply.

Lactacystin and LLnL have different effects on the maturation of HLA-A2 in a TAP-deficient cell line

HLA-A2 is expressed on the surface of TAP-deficient T2 cells. These molecules are mostly associated with peptides derived from leader sequences (2, 3). The generation of these leader peptides is expected to be proteasome-independent. Peptide aldehyde inhibitors have been shown to inhibit the generation of some of these leader peptides (15). We wished to know if lactacystin also has such effects. The maturation of HLA-A2 proceeds normally in T2 cells (Fig. 3*A*, *lanes 1–4*), which, we assume, is largely supported by TAP-independent leader peptides. In agreement with the previous report (15), we found LLnL significantly retarded this maturation (*lanes 9–12*). In contrast, lactacystin has a much lesser effect (*lanes 5–8*). These data are quantitatively displayed in Figure 3, *B* and *C*, where it is shown that both the percent of total endo-H-resistant forms of A2 as well as the absolute amount of endo-H-resistant A2 is decreased by LLnL but is only marginally affected by lactacystin. Both inhibitors delay the maturation of HLA-A2 in C1R.A2 cells (Fig. 3, *F–H*), in which most peptides are TAP-dependent. That a significant amount of HLA-A2 is processed into the mature form in lactacystin-treated C1R.A2 cells is not surprising, because leader peptides have been found to associate with HLA-A2 in normal cells (2). It is also expected that LLnL, which partially inhibits the generation of leader peptides, inhibits the maturation of HLA-A2 in C1R.A2 cell more potently than lactacystin. As a peptide-independent glycoprotein control, the transferrin receptor was tested for its maturation in the presence of these inhibitors. Neither of these inhibitors significantly affect the maturation of the transferrin receptor (Fig. 3, *D* and *E*). Our results suggest that the action of lactacystin is more specific than that of LLnL, and the inhibition of class I Ag presentation by lactacystin can be largely attributed to the inhibition of peptide generation in the cytosol by the proteasome.

FIGURE 2. The maturation of class I molecules is inhibited by lactacystin and LLnL. *A*, RMA cells were preincubated in methionine-free medium containing no inhibitor (*lanes 1–3*, ■), lactacystin (*lanes 4–6*, ●) or LLnL (*lanes 7–9*, ▼) for 90 min. RMAS cells (*lanes 10–12*, ▲) were preincubated without inhibitor. Cells were pulse labeled with [^{35}S]methionine for 20 min and chased for up to 3 h in the continued absence or presence of inhibitors. At the indicated times, cells were lysed and D^b molecules were precipitated with mAb 28-14-8s. All immunoprecipitates were treated with endo-H before SDS-PAGE analysis. The percentage of endo-H-resistant forms in total D^b molecules was plotted (*B*). *C* and *D*, LFA-1 molecules were precipitated with mAb FD441.8 from RMA cells treated without (*lanes 1–4*, ■) or with lactacystin (*lane 5–8*, ●) or LLnL (*lanes 9–12*, ▼). The α-chains of LFA-1 are shown and analyzed quantitatively. *E* and *F*, L^d molecules were precipitated with mAb 28-14-8s from control (*lanes 1–3*, ▲) or inhibitor-treated (lactacystin, *lanes 4–6*, ●; LLnL, *lanes 7–9*, ▼) L^d-transfected L cells.

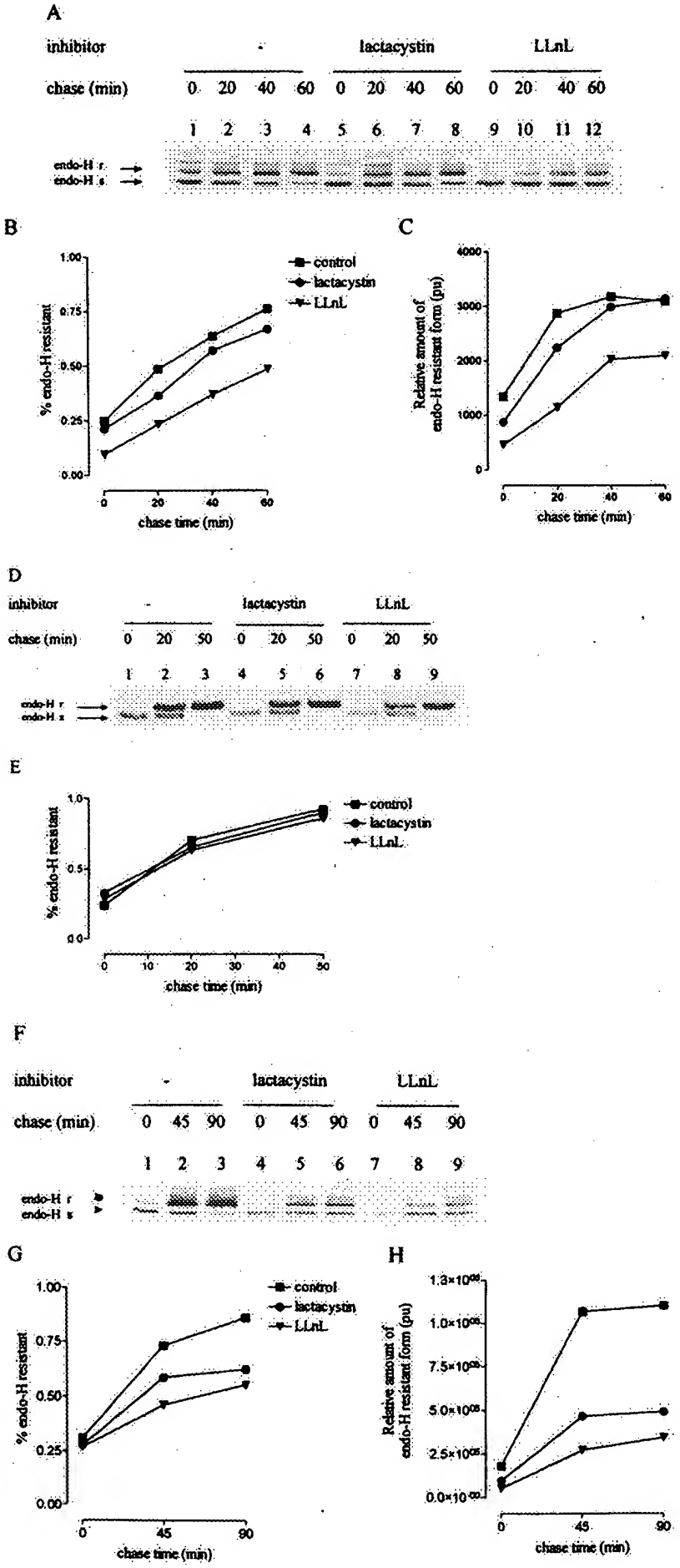


FIGURE 3. Effects of proteasome inhibitors on the maturation of HLA-A2 in cell lines with or without functional TAP. T2 (*A–E*) or C1R.A2 (*F–H*) cells were treated with inhibitors and pulse chased as described in the legend to Figure 2. HLA-A2 was precipitated with mAb MA2.1 (*A, F*). The quantitative data are plotted either as the percentage of endo-H-resistant forms (*B, G*) or the total activity detected in the endo-H-resistant bands (*C, H*). The transferrin receptor was precipitated with mAb 1G12 (*D, E*).

Lactacystin does not block the maturation of Qa-1^b

The murine class Ib molecule Qa-1^b presents an endogenous peptide termed Qdm that is derived from the leader sequences of D-end class I molecules (except D^k) (4). The presentation of the Qdm peptide is TAP dependent. To test if this reflects the requirement for the proteasome to process this peptide, Qdm-driven Qa-1^b maturation was analyzed in the presence of proteasome inhibitors. L-g37 is derived from a fibroblast cell line of H-2^k background by stable transfection of Qa-1^b. Qa-1^b matures to a certain degree in this Qdm$^-$ cell line (Fig. 4*A*, *lanes 1–4*). Infection with vaccinia virus carrying an HPV insert (*lanes 5–8*) or wild-type vaccinia virus (data not shown) abrogates the background maturation. The leader sequence of D^d contains the Qdm epitope. Infection of L-g37 cells with recombinant vaccinia containing a D^d insert (Vac-D^d) results in an accelerated maturation of Qa-1^b (*lanes 9–12*). This maturation is driven by the D^d insert because vaccinia with an irrelevant (HPV) insert does not have such an effect (*lanes 5–8*). Lactacystin does not affect the generation of the mature form of Qa-1^b in Vac-D^d-infected cells (*lanes 13–16*). In contrast, LLnL does have an inhibitory effect (*lanes 17–20*). Neither inhibitor has an effect on vaccinia infection and the synthesis of D^d molecules as determined by a [^{35}S]methionine pulse followed by immunoprecipitation of D^d from Vac-D^d-infected cells (data not shown). These data are also presented quantitatively where it appears that both lactacystin and LLnL inhibit the percentage of endo-H-resistant forms (*panel B*). However, the total amount of the endo-H-resistant form of Qa-1^b is not decreased by lactacystin (*panel C*), rather the disappearance of the endo-H-sensitive form is delayed (*panel A*, compare *lanes 15*, *16*, *19*, and *20* with *lanes 11* and *12*), suggesting that both lactacystin and LLnL play a role in inhibiting the degradation of Qa-1^b. To exclude the possibility that this is a particular finding relates to the virus infection, we further examined the maturation of Qa-1^b in Qdm$^+$ lymphoblasts (Fig. 4, *D–F*). Previous studies indicate that the Qdm peptide is the dominant peptide presented by Qa-1^b in Qdm$^+$ lymphoblasts (25). The maturation pattern of Qa-1^b may largely reflect the availability of the Qdm peptide. As seen in *D–F*, the effect of lactacystin and LLnL in lymphoblasts is similar to those in vaccinia-infected cells; i.e., lactacystin does not inhibit the maturation of the endo-H-resistant form of Qa-1^b. These data suggest that the generation of the Qdm peptide is proteasome independent.

LLnL but not lactacystin inhibits the surface presentation of the Qdm peptide

To more specifically characterize the role of the proteasome in the processing of the Qdm peptide, the surface presentation of this peptide was directly measured by a peptide specific CTL assay. Two types of Qa-1^b-specific CTL clones, Qdm-dependent (D) and Qdm-independent (I), have been established from alloreactive CTL (4). 3C9 is a Qdm-D clone which recognizes Qa-1^b only when it binds the Qdm peptide. 5D2 is a Qdm-I clone that recognizes both Qdm-bound Qa-1^b as well as Qa-1^b on cells of H-2^k background (Qdm$^-$). L-g37 (H-2^k) control cells can be readily lysed by 5D2 but not 3C9 CTL (Fig. 5, *A* and *B*). Upon infection with Vac-D^d, L-g37 is sensitized for lysis by 3C9 CTL (Fig. 5*B*), and the lysis mediated by 5D2 CTL is enhanced because these CTL can recognize the Qdm peptide (4). Including lactacystin during the vaccinia infection only slightly inhibits the sensitization of target cells for 3C9 lysis and does not inhibit the enhanced lysis mediated by 5D2 CTL, indicating the Qdm peptide is still generated and presented on the surface by Qa-1^b molecules. Further, adding back the Qdm peptide does not enhance the lysis of the lactacystin-treated target cells. On the other hand, LLnL does block the lysis by 3C9 cells as well as partially inhibit the lysis mediated by 5D2 cells. Since the lysis of LLnL-treated cells can be restored after pulsing with the synthetic Qdm peptide, LLnL probably blocks the generation of Qdm and other Qa-1^b-dependent epitopes rather than altering the susceptibility of target cells for CTL lysis. Vac-HPV cannot sensitize L-g37 for 3C9 lysis.

As a control for showing the inhibition of epitope generate by lactacystin, the generation of the $OVA_{257-264}$ (SIINFEKL) epitope is blocked by both inhibitors as determined by an acid wash/recovery assay (Fig. 5*C*). These results firmly established that the processing of Qdm is proteasome independent.

Discussion

Lactacystin is the most specific proteasome inhibitor known so far. It is a useful tool for the elucidation of the involvement of the proteasome in certain biologic processes (26, 27). The proteasome has long been implicated as the major proteolytic system responsible for the generation of peptides presented by class I molecules (8). Several groups have addressed this question using peptide aldehyde inhibitors and found that certain members of this class of inhibitors block class I Ag presentation (12–14). These inhibitors potently inhibit the proteasome as well as some other proteases such as calpain and cathepsin B (12). Although there is a good correlation between the inhibition of class I Ag presentation and in vitro inhibition of the purified proteasome, the contribution of their nonspecific activities is still largely unpredictable. This concern is further highlighted by the recent finding that some of these peptide aldehydes can inhibit the presentation of leader peptides in TAP-deficient cell lines (15). Here, using lactacystin we more specifically investigated the role of the proteasome in class I Ag presentation. Our results support the idea that the majority of peptides presented by class I molecules are generated by the proteasome. Inhibition of the proteasome with lactacystin can block the maturation of class I molecules, which is a typical manifestation of deficient peptide supply. More importantly, the maturation of a peptide-independent glycoprotein is not affected by the same treatment. For class I molecules D^b and L^d, the effect of lactacystin is indistinguishable from that of LLnL, which has been shown to be capable of inhibiting class I Ag presentation with multiple experimental systems (12, 15). We noted that the effects on the two class I alleles tested are different in degree. Whereas the maturation of L^d is almost completely blocked by these inhibitors, the maturation of D^b is only partially inhibited. This could be attributed to either the difference in their abilities to bind proteasome-independent peptides or the different efficiencies of the ER retention system to retain empty class I molecules of different alleles. D^b molecules can escape from the ER more efficiently without sufficient peptide supply, as illustrated in RMAS cells.

In the TAP-deficient cell line T2, surface expression of HLA-A2 is much less severely impaired than that of other class I alleles. The surface expressed HLA-A2 molecules in these cells are peptide loaded. The majority of the peptides presented are derived from leader sequences, which are accessible to the ER lumen through the protein translocation machinery on the ER membrane (2, 3). These peptides are assumed to be processed within the ER and are expected to be proteasome independent. Therefore, this model can be used to differentiate cytosolic (proteasomal) processing and ER processing. Hughes et al. (15) found that two peptide aldehyde inhibitors (LLnL and Z-LLF-CHO) can inhibit the maturation of HLA-A2 in the TAP-deficient cell line .174 and decrease the amount of signal peptides presented on the surface of these cells. Our data confirms that LLnL slows the maturation of HLA-A2 in TAP-deficient T2 cells. In contrast, lactacystin has a much lesser

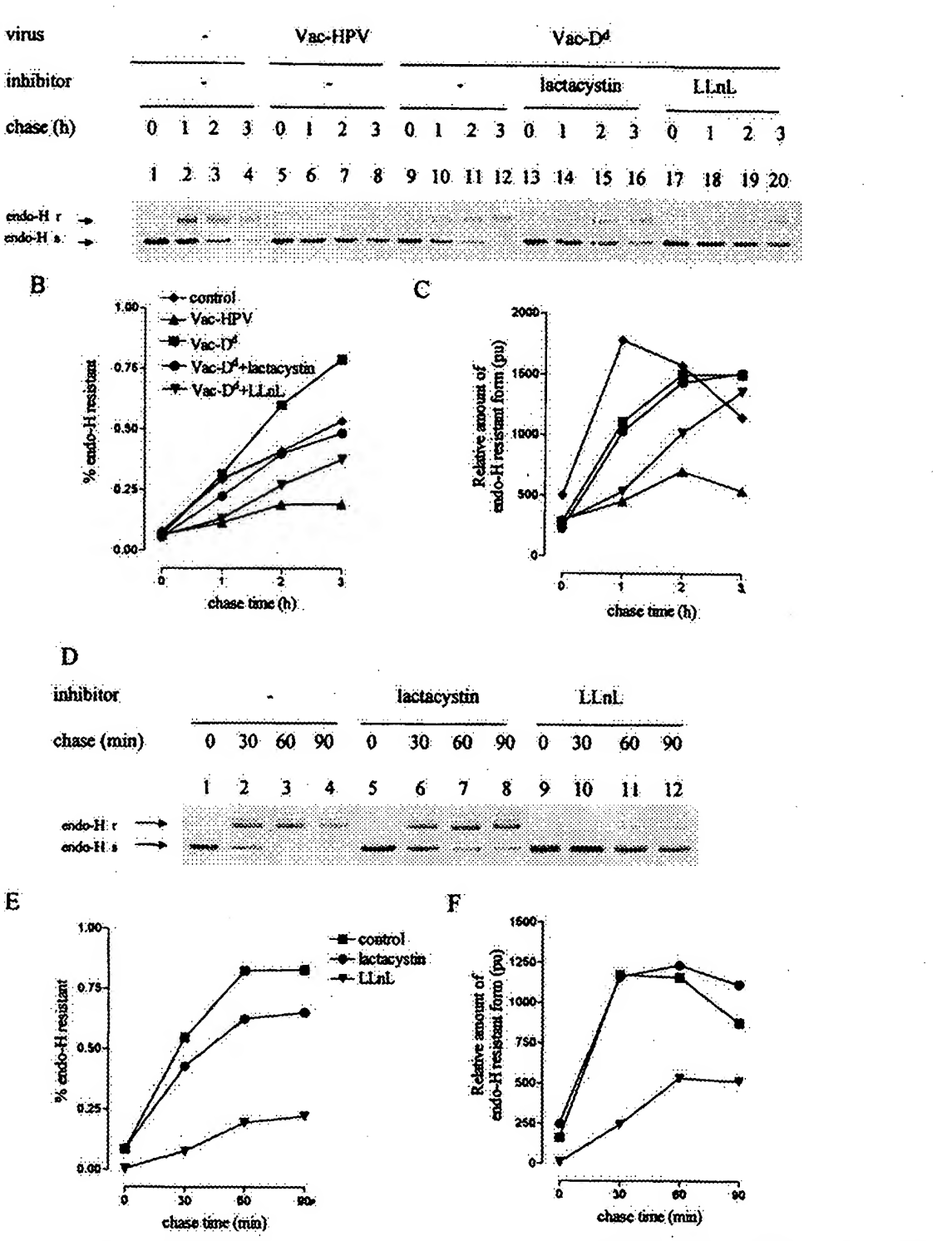


FIGURE 4. Lactacystin does not block the maturation of Qa-1^b. (*A–C*) L-g37 cells were incubated with 50 plaque-forming units/cell of Vac-HPV (*lanes 5–8*) or Vac-D^d (*9–20*) for 2 h. Unbound virus was removed, and methionine-free medium with or without inhibitors was added. After another 90′ of incubation, cells were pulse-labeled with [^{35}S]methionine for 20 min and chased for various times. Qa-1^b molecules were precipitated with a rabbit anti-Qa-1^b cytoplasmic region specific Ab. Control cells (*lanes 1–4*) were neither infected with virus or treated with inhibitors. (*D–F*) Splenocytes from BALB/c (H-2^d, Qa-1^b) mice were stimulated with Con A for 2 days. Pulse chase and immunoprecipitation of Qa-1^b were performed in the absence or presence of inhibitors as described above. All immunoprecipitates were separated with SDS-PAGE following the digestion of endo-H. The quantitative data are plotted either as the percentage of endo-H-resistant forms (*B, E*) or the counts of the endo-H-resistant bands (*C, F*).

effect, indicating its higher specificity. However, a small but consistent inhibition by lactacystin could be found from quantitative analysis. We propose that two possibilities may cause this. First, a minor portion of peptides presented by HLA-A2 in TAP-deficient cells may be proteasome dependent. Recently, peptide epitopes derived from EBV latent membrane protein 2 are found to be presented by HLA-A2 in T2 cells (28). This molecule is a multiple membrane-spanning protein with cytosolic N- and C-terminal domains. Two TAP-independent epitopes are located in the transmembrane domains. Although the epitopes themselves are probably generated within the

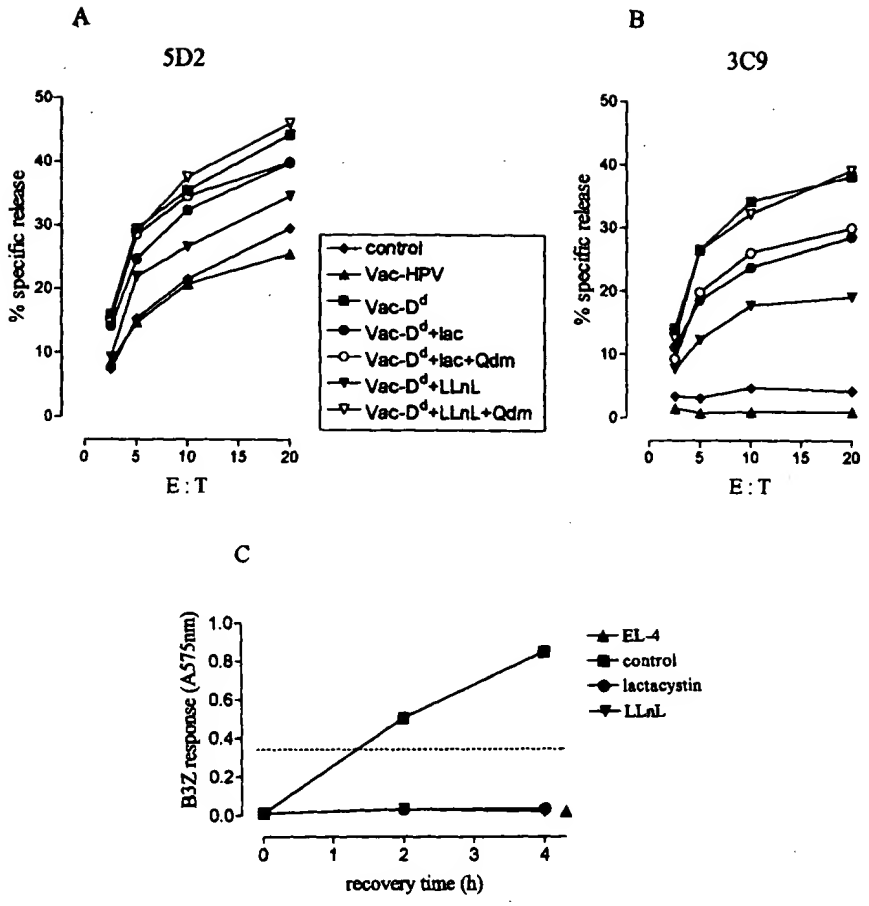


FIGURE 5. Effects of lactacystin and LLnL on the presentation of the Qdm peptide to CTL clones. L-g37 cells were infected with 10 plaque-forming units/cell of Vac-HPV (▲) or Vac-D^d in the absence (■) or presence of lactacystin (●) or LLnL (▼) for 3.5 h. In the last 30' of the viral infection, BFA was added. The sensitization of target cells for lysis by Qdm-I clone 5D2 (*A*) or Qdm-D clone 3C9 (*B*) was tested with a 4-h CTL assay in the presence of BFA. LLnL (▽) or lactacystin (○)-treated targets were pulsed with 100 ng of synthetic Qdm peptide (AMAPRTLLL) before the CTL assay. *C*, E.G7-OVA cells were cultured at pH 3.1 for 3 min at 25°C. After recovering in the complete medium in the absence (■) or presence (●) of lactacystin or LLnL (▼) for various times, cells were fixed with 1% paraformaldehyde. 5×10^4 fixed cells were incubated with 5×10^4 B3Z T cells for 5 h. The LacZ activity induced in the B3Z cells were measured with the CPRG substrate. Nontransfectant EL-4 cells (▲) and E.G7-OVA cells without acid treatment (dotted line) were fixed and directly used as APC.

ER, the degradation of the protein may initiate on the cytosolic side by the proteasome. The generation of such peptides may require the coordinated processing of the proteasome and ER proteases. Second, we cannot exclude the possibility that lactacystin affects other proteolytic enzyme systems to a small extent.

One original purpose of our studies was to elucidate the processing mechanism of the TAP-dependent leader peptide Qdm. One possibility of the TAP dependence for the presentation of this epitope could be that proteasome-dependent enzymatic activity is required for generation of the precise epitope. We used both a biochemical and functional approach to address this issue. To use a biochemical approach to characterize an individual peptide, we must be certain that the biochemical behavior of the class I molecule is dominated by the peptide in question. Without the knowledge of which peptides are responsible for the surface expression of Qa-1^b in unmanipulated L-g37 cells, we found wild-type vaccinia infection markedly suppresses the maturation of Qa-1^b. When Qdm is introduced under this low level endogenous peptide supply, the resulting maturation pattern of Qa-1^b reflects the availability of the Qdm peptide. Another cell type that we used is Con A-stimulated splenocytes. From previous experiments, we know that in Qdm^+ lymphoblasts, Qdm is a dominant peptide that occupies most surface Qa-1^b molecules (25). Our results demonstrate that in the presence of lactacystin Qa-1^b can still be processed into the mature form, whereas LLnL has an inhibitory effect. This strongly suggests that most peptides presented by Qa-1^b (probably Qdm) do require a certain kind of processing that can be inhibited by LLnL, but this processing is proteasome independent. In these experiments, we consistently observed that the disappearance of the endo-H-sensitive form of Qa-1^b was slowed by lactacystin. A possible explanation for this observation is that lactacystin can inhibit the degradation of Qa-1^b molecules. Given the fact that lactacystin is a proteasome specific inhibitor, this could occur in two circumstances. First, the degradation of Qa-1^b in the ER may involve the proteasome. It has been reported that the degradation of membrane proteins in the ER can be initiated from the cytosolic side by the proteasome (26). Class I molecules can be dislocated into the cytosol and degraded by the proteasome in the CMV-infected cells (27). Second, Qa-1^b may associate with other peptides with a low affinity. These low affinity peptide-associated Qa-1^b molecules can be transported to the cell surface and then are quickly degraded. The generation of these low affinity peptides may be proteasome dependent and can be blocked by lactacystin. Thus, Qa-1^b molecules are retained for a longer time in the ER. Since Qa-1^b molecules loaded with low affinity peptides cannot accumulate on the cell surface, their depletion does not affect the amount of mature form of Qa-1^b. To definitively establish whether the presentation of the Qdm epitope required proteasomal activity, we tested the ability of the Qdm-D CTL clone 3C9 to recognize Qdm on Qdm^- cells that were infected with a recombinant vaccinia virus that contained the Qdm epitope. These experiments showed that lactacystin had no inhibitory effect on the appearance of the epitope. On the other hand, inhibition was noted with LLnL suggesting that other proteases, perhaps localized in the ER, are required for epitope generation.

Our findings do not support the hypothesis that the TAP dependence of the Qdm peptide is due to the requirement for the proteasome. Other explanations may account for this observation. Leader peptides are embedded in the ER membrane after cleavage

by signal peptidase. Lyko et al. (29) found that these leader peptides can be further processed, and some resulting fragments can be released into the cytosol. If the fragment containing the Qdm epitope is exclusively released into the cytosol, it may need the TAP transporter to enter the class I pathway. If this is the case, we cannot exclude that in the normal situation the proteasome does participate in the processing of these fragments. The altered maturation pattern we observed in the presence of lactacystin may reflect the kinetic differences between the proteasome and ER proteases in generating the Qdm peptide. Another possibility is that TAP may be involved in the assembly of Qa-1^b with Qdm. Introduction of the Qdm peptide directly into the ER in TAP-deficient cell lines may tell us whether this is a restriction factor.

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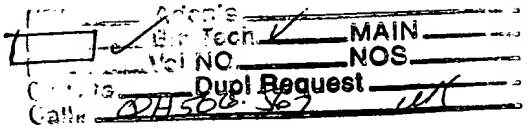

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V 278 COMPLEMENTARY MUTANTS IN MHC CLASS II/PEPTIDE COMPLEX ASSEMBLY Steven P. Fling and Donald A. Pious, Department of Pediatrics, University of Washington, Seattle, WA 98195.

Our lab has previously mapped to the MHC a gene involved in class II antigen processing and presentation, using a set of mutant B-LCL derived by mutagenesis and immunoselection of B-LCL 8.1.6; 8.1.6 contains an 800 kb hemizygous deletion within the class II region of the HLA. The lesion in these mutants results in a failure to form normal intracellular class II/peptide complexes which is manifested by: 1) conformationally altered, unstable class II dimers; 2) association of Ii chain derived peptides with DR; and 3) an inability to present whole exogenous antigens. Available evidence suggests that the affected gene maps to a 230 kb interval within the MHC hemizygous deletion of 8.1.6.

We describe here the isolation of a set of mutants which manifest global alterations in class II conformation and stability similar to those seen in the 8.1.6 derived mutants. The new mutants, which were derived by mutagenesis and immunoselection of a B-LCL which contains a different hemizygous deletion of chromosome 6p, are complementary with the 8.1.6 derived mutants in somatic cell hybrids. These results suggest the possibility of an additional locus whose product is involved in the assembly of class II/peptide complexes. Alternatively, complementation in these mutants could occur by intragenic means, perhaps by molecular complementation in a homomultimeric protein..

V 279 GENERAL AND UNIQUE PEPTIDE-SPECIFICITIES EXHIBITED BY TRANSPORTERS ASSOCIATED WITH ANTIGEN PROCESSING (TAP) FROM MOUSE, RAT, AND HUMAN. Lars Frankson, Elisabeth Wolpert and Klas Kärre, Microbiology and Tumorbiology Center, Laboratory of Tumor Biology, Karolinska Institutet, S-17177 Stockholm, Sweden.

Transporters associated with antigen processing , TAP, are considered to transport short peptide fragments from the cytosol to the lumen of the endoplasmic reticulum, where they are loaded on MHC class I molecules. In vivo the lack of functional TAP2 molecules allow tumor cells to escape from T cell mediated immune surveillance. In vitro the TAP molecules have been shown to influence the rate and spectrum of peptides being transported over the ER membrane. Here we address the questions how different TAP molecules i) affect the tumorigenicity of tumor cells in vivo, and ii) affect the peptide repertoire presented by MHC class I molecules at the cell surface. Ad i), three independent TAP2 transfectants (mouse, rat, and human) of the antigen presentation defect mouse lymphoma RMA-S were inoculated s.c. over a minor histocompatibility barrier, MiHa, and monitored for outgrowth. All three TAP2 transfectants grew out and were subsequentially rejected equally well as the wild type line RMA, whereas the non-transfected RMA-S grew out and killed the A.BY mice. Ad ii), naturally processed peptides were eluted under acid conditions from either intact cells or immuno-affinity purified MHC class I molecules of the three RMA-S TAP2 transfectants. The eluates were analysed on reverse phase HPLC and capillary electrophoresis, CE. In addition to this a functional identification of epitopes was performed utilising cytotoxic T lymphocytes specific for either three MiHa, or a tumor antigen. The HPLC- and CE-profiles differed between the three TAP2 transfectants. However, the three MiHa and the tumor antigen previously characterised on the wild type line RMA were identified by the CTL in each peptide-eluate. The data suggests a general capacity of the TAP molecules to transport certain peptides, among which the CTL epitopes screened for here are found. In addition to this each TAP molecule appear to have a unique fine specificity for peptides as indicated by the differences in the HPLC-and CE-profiles, though further chracterisation of the material remains to be done. Finally, the restored rejectability by the TAP2 transfections pinpoints the decisive role that one single gene can have on T cell mediated immune surveillance.

V 280 INTERFERON-γ INDUCIBLE PROTEASOME SUBUNITS REPLACE CONSTITUTIVELY EXPRESSED SUBUNITS Klaus Früh, Per A. Peterson and Young Yang
The Scripps Research Institute, Dept. of Immunology, 10666 North Torrey Pines Road, La Jolla, CA 92037

The proteasome is thought to be the cytoplasmic protease responsible for the generation of peptides presented by MHC class I molecules. Two subunits, LMP2 and LMP7, of the proteasome are encoded in the MHC class II region of the genome and the expression of LMP2 and LMP7 can strongly be induced by IFN-γ. We have observed previously that this induction not only leads to the enhanced presence of LMP2 and LMP7 in the proteasomal complex, but also to the disappearence of certain other subunits. We have now further characterized the events taking place during the incorporation of LMP2 and LMP7 into the proteasome. Our data indicate that both LMP2 and LMP7 replace a disinct other subunit. The respective subunits are constitutively expressed and highly homologous to the MHC-encoded ones. The results will be discussed with regard to proteasome function and antigen processing.

V 281 PATHWAYS INVOLVED IN ANTIGEN PROCESSING FOR MHC CLASS II-MEDIATED ANTIGEN PRESENTATION, Anand M. Gautam and Hugh O. McDevitt#, Human Genetics Group, John Curtin School of Medical Research, The Australian National University, Canberra, ACT 2601, Australia and #Department of Microbiology and Immunology, Stanford University, California 94305, USA.

Generally, major histocompatibility complex (MHC) molecules present processed foreign peptides to specific T cells. The recently described genes, LMP2, LMP7 (subunits of proteosome complex) and TAP1, TAP2 (ATP-dependent transporters) have been shown to regulate antigen processing for MHC class I molecules. However, such genes have not been defined for MHC class II molecules. We have generated antigen processing mutants for MHC class II-mediated antigen presentation. These mutants fail to present whole antigens but retain the ability to present peptides to antigen-specific and MHC class II-restricted T cell clones. This indicates that these cells have certain defects in antigen processing. Our aim is to utilise these mutants to study pathways and genes which may control MHC class II-mediated antigen processing. The putative gene (s) involved in antigen processing for class II molecules have been localised in the MHC class II region. Invariant chain (Ii) prevents binding of endogenous peptide to MHC class II molecules in the endoplasmic reticulum and hence dissects MHC class II from MHC class I pathway of antigen presentation. MHC class II molecules in processing mutants are predominantly occupied with an Ii peptide, called CLIP (class II-associated Ii peptide). We have also developed an experimental system to determine binding characteristics of Ii to MHC class II molecules by utilizing the CLIP.

Presentation by a Major Histocompatibility Complex Class I Molecule of Nucleoprotein Peptide Expressed in Two Different Genes of an Influenza Virus Transfectant

By Hideki Isobe,* Thomas Moran,* Shengqiang Li,* Aideen Young,‡ Stanley Nathenson,‡ Peter Palese,* and Constantin Bona*

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Summary

Major histocompatibility (MHC) class I glycoproteins are specialized to present to CD8+ T cells, peptides that originate from proteins synthesized within the cytoplasm. Conventional killed vaccines are unable to get into the cell cytoplasm and therefore fail to expand the CD8+ T cell population. We have created a novel influenza transfectant virus, R10, which carries an immunogenic peptide from the nucleoprotein (NP) of PR8 influenza virus in its hemagglutinin (HA) and another similar peptide in its HK influenza virus NP. The two peptides are both presented by H-2D^b and bind with approximately equal affinity. They can compete with one another for binding to H-2D^b. Yet in cells infected with R10, both peptides are presented efficiently enough to expand the respective cytotoxic T lymphocyte (CTL) precursors in vivo and to serve as targets for CTL lysis in vitro. It has been proposed that proteins bearing signal sequences may be processed by a transporter-independent pathway. To investigate this, we infected the transporter-deficient cell line RMA-S with the R10 virus to see if the NP peptide expressed by the HA would be presented. The result shows that even the presence of a signal peptide in the HA does not overcome the lack of a transporter function, suggesting that the presentation of both peptides is dependent on functional transporter proteins. Our data also suggest the feasibility of creating by genetic engineering, recombinant vaccines expressing multiple epitopes that can effectively stimulate a cellular immune response.

CTL recognize short peptides derived from the processing of intracellular proteins that are presented at the cell surface by class I molecules of the major histocompatibility locus (1, 2). The isolation of naturally processed peptides from class I antigens has shown that for each allele the peptides exhibit a "motif" characterized by a few amino acids, and that these residues are crucial for binding to MHC antigens (3–7). This concept was strengthened by the resolution of the crystal structure of class I molecules showing that the consensus residues dock within pockets of the antigen-binding cleft (8–10).

Sensitization of cells with synthetic peptides showed that MHC molecules can bind and present specific peptides as well as analogues that differ in structure and length (10, 11). Whereas numerous studies have focused on the recognition of natural peptides and synthetic analogues by CD4 T cells (12–14), there is limited information on the effects of analogues on the presentation of endogenous viral peptides associated with class I antigens to CD8+ T cells (15). These questions are particularly important for the development and the use of recombinant vaccines containing multiple epitopes.

Our study was aimed at investigating the presentation of two influenza virus nucleoprotein (NP)[1]-derived peptides that bind to the same allele (i.e., D^b). Conceptually, these two NP peptides may be considered to be analogues. To carry out this study, we took advantage of a new reverse genetics approach allowing the expression of foreign epitopes or antigens by influenza virus. This method is based on the rescue of cDNA-derived RNA into infectious viral particles (16). Using this technique it was possible to express in a hemagglutinin (HA) molecule the epitopes of HA of different virus

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; HA, hemagglutinin; NP, nucleoprotein; SP, spleen cell; TAP, transporter-associated with antigen processing.

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subtypes (17), an epitope derived from the V_3 loop of the HIV-1 glycoprotein (gp120) protein (18), and the epitope of the circumsporozoite protein of *Plasmodium yoelii* (19). For the present study, we created a chimeric influenza virus by inserting into antigenic site E of the HA of influenza A/WSN/33 virus, a nonamer peptide derived from the NP of influenza A/PR/ 8/34 virus (PR8). The chimeric *HA* gene was then transferred into a virus that derives the remaining seven RNA segments from influenza A/HK/8/68 virus (HK). Therefore, our chimeric virus (R10) expresses the PR8-NP peptide within the HA and the HK-NP peptide from its NP protein. PR8-NP and HK-NP peptides share the same anchoring motifs and differ by only two amino acid residues (20). This influenza virus transfectant expressing both peptides was used to study: (*a*) whether antigenic competition hinders the efficient presentation of two peptide analogues presented to CTLs by the same allele; and (*b*) how the transporter-associated with antigen processing (TAP) dependent transport system (21) affects the efficiency with which peptides are presented from proteins with and without signal sequences.

Materials and Methods

Mice. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Viruses. Influenza A/PR/8/34 and A/HK/8/68 viruses were grown in embryonated eggs, whereas the chimeric influenza virus R10 was grown in Madin Darby canine kidney (MDCK) as previously described (16).

Cell Lines. EL-4, RMA, and RMA-S cells (kindly donated by Dr. J. Yewdell, National Institutes of Health, Bethesda, MD) were grown in DMEM medium supplemented with 10% FCS and were used as targets for the cytotoxic assays.

Peptides. Peptide ASNENMETM corresponding to amino acid residues 366-374 of the influenza virus A/PR/8/34 NP and ASNENMDAM corresponding to amino acid residues 366-374 of the influenza A/HK/8/68 NP were synthesized in the Department of Pharmacology, Mount Sinai Medical Center and were purified by HPLC. Two peptides corresponding to amino acid residues 229-237 (CKGVNKEYL) and 489-497 (QGINNLDNL) of the SV40 T antigen were a gift from Dr. J. Yewdell.

Construction of the Transfectant Virus TE Expressing the PR8-NP Epitope. Plasmid pTE was constructed by replacing the BstEII-HindIII fragment of pT3/WSN-HA containing the influenza A/WSN/33 virus *HA* gene (19) with a PCR product, in which the nucleotide sequence encoding seven amino acid residues at the antigenic site E of the HA was replaced by the sequence encoding the PR8-NP peptide ASNENMETM. Rescue of infectious virus carrying the pTE plasmid–derived RNA was done as described (17). The nucleotide sequence encoding the PR8-NP peptide was confirmed by direct sequencing of the viral HA segment as previously described (17). All other genes of the virus are derived from influenza A/WSN/33 virus.

Generation of the Reassortant R10 Virus. The reassortment of viruses has been done as described (22). Briefly, Madin Darby canine kidney (MDCK) cells were infected with influenza HK virus and UV-irradiated TE virus. The supernatant of the infected cells was used for plaque assay in the presence of anti-HK antiserum. From 10 individually isolated plaques, R10 was genotyped and shown to contain its *HA* gene derived from the TE virus and its remaining genes from HK virus (Fig. 1).

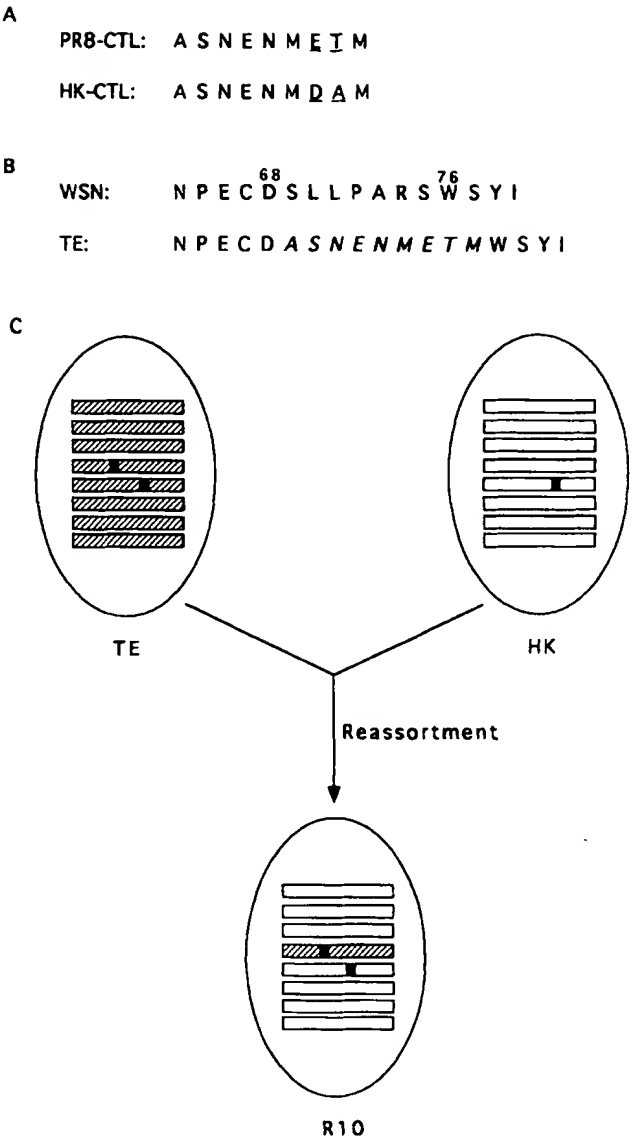

Figure 1. Construction of the transfectant TE virus and the reassortant virus R10. (*A*) The amino acid sequences of the D^b-restricted NP-specific CTL epitopes of influenza PR8 and HK viruses. Differences between the peptides are underlined. (*B*) The transfectant virus TE was designed to express the PR8 virus–specific CTL epitope in site E of the influenza A/WSN/33 virus HA. It was constructed by replacing the 7-amino acid peptide of the HA with the 9-amino acid CTL peptide (indicated in *italics*). (*C*) The reassortant virus R10 was generated by mixed infection of the transfectant TE virus and the influenza A/HK/8/68 virus. It derives the *HA* gene (RNA segment 4) from the TE virus and the remaining genes from HK virus. The CTL epitopes of the NP proteins of PR8 and HK viruses are indicated in black or gray boxes, respectively. It should be noted that the NP-specific PR8 CTL epitope is also present in the NP of the TE virus but not in reassortant virus R10 which contains HK virus NP segment.

Induction of CTL Response. C57BL/6 mice were immunized intraperitoneally with 0.2 ml of a viral suspension containing 10^7 PFU of PR8, HK, or R10 viruses. 7 d later, spleen cells (SC) were obtained and restimulated in vitro for 5 d with irradiated SC alone or coated with PR8-NP or HK-NP peptides or SC infected with PR8, HK, or R10 viruses as previously described (23). CTL clones

were obtained by limiting dilution (1 or 3 cells/well) and stimulated with SC coated with NP peptides.

Cytolysis Assay. EL-4, RMA, or RMA-S cells coated with PR-NP or HK-NP peptides or infected with PR8, HK, or R10 viruses were labeled with $Na^{51}CrO_4$ (100 μCi/10^6 cells) for 1 h at 37°C. After two washings, the cells were transferred to V-bottom 96-well plates, the effector cells were added, and the mixture was incubated at 37°C in 7% CO_2. 4 h later, the supernatant was harvested and counted. The maximum chromium release was determined by incubating the cells with 1% NP-40. The percentage of specific lysis is calculated according to the following formula: 100 × [(cpm samples − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release)].

Immunostaining. RMA-S cells were infected with 10^7 PFU of PR8 or R10 viruses. The presence of HA on the surface was detected by immunostaining as previously described (24). For immunostaining of cells, mAbs PY211 and 2G9 specific for the HA of PR8 virus and the HA of WSN virus, respectively, were used. As a negative control, mAb PY206 specific for the HA of X31 virus was used. Identical samples of infected cells were analyzed for hemadsorption.

Results

Recognition of PR8 and HK-NP Peptides. The two peptides which are derived from the NPs of influenza PR8 and HK viruses and bind to the D^b MHC glycoprotein, differ by two amino acids. The PR8-NP peptide has glutamic acid and threonine at positions 7 and 8 respectively, whereas the HK-NP peptide has aspartic acid and alanine in these positions (3). CTLs are able to discriminate between these subtle differences, as shown by our ability to generate NP peptide-specific CTL clones from C57BL/6 mice immunized with PR8 or HK viruses. Upon in vitro culture of splenic lymphocytes with PR8-NP or HK-NP peptides, the cells were cloned at 1–3 cells/well and expanded for several rounds. The data depicted in Fig. 2 show that PR8-NP–specific CTLs lysed EL-4 cells coated with PR8-NP but not with HK-NP peptide, and that HK-NP–specific CTLs lysed only EL-4 cells coated with HK-NP peptide. Whereas 0.01 μM of PR8-NP and HK-NP peptides was required to sensitize EL-4 target cells for significant lysis by CTL clones, 1 μM of peptide was necessary to reach the plateau of sensitization (data not shown). Similar results were obtained with RMA and RMA-S cells (data not shown).

Competition of Binding of PR8-NP and HK-NP Peptides to Surface D^b Glycoprotein. A potential drawback to the use of virus transfectants, as vaccines expressing multiple peptides specific for the same allele, is that intramolecular competition may occur with one peptide being preferentially presented over others. The PR8-NP and HK-NP peptides differ by only two amino acid residues, and since the anchoring residues are identical, they may be considered to be analogues. Thus, if intramolecular competition occurs, it should be demonstrable using these peptides. To confirm that they had this capability, we studied the competition at the level of peptide presentation by simultaneously incubating constant amounts of PR8-NP peptide with variable amounts of HK-NP peptide and vice versa. When we incubated EL-4 cells with 10 μM PR8-NP peptide and various amounts (10–100 μM) of HK-NP peptide, little inhibition of lysis of EL-4 cells by PR8-NP–specific CTL was observed. Only a weak inhibition was also observed when 10 μM HK-NP peptide was incubated with various amounts of PR8-NP peptide and HK-NP specific CTLs (data not shown). In contrast, a strong inhibition was observed when only 1 μM of PR8-NP peptide was simultaneously incubated with the HK-NP peptide or with peptides derived from SV40 virus which are recognized by $CD8^+$ T cells in association with the D^b MHC class I glycoprotein (see Fig. 3 *A*). This was also true when EL-4 cells were simultaneously incubated with 1 μM HK-NP peptide and various amounts of PR8-NP or the SV40 peptides (Fig. 3 *B*). These results suggest that PR8-NP and HK-NP peptides display a similar affinity for the D^b class I MHC glycoprotein and have the potential to compete with one another for occupancy in the D^b binding site.

We incubated the target cells for 30 min with PR8-NP or HK-NP peptides, and then washed and incubated them for 2 h with various amounts of analogues. No inhibition of lysis of EL-4 cells by PR8-NP or HK-NP specific CTL was observed (data not shown). These results suggest that the two peptides have similar affinities for the MHC molecules.

Presentation of the PR8-NP Peptide Expressed within the HA of Influenza Virus Transfectant R10. In preliminary experi-

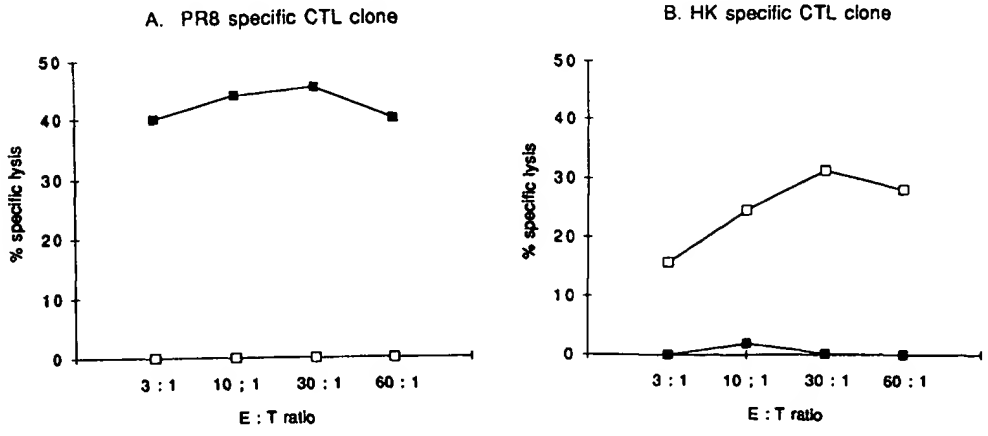

Figure 2. Specific lysis of EL-4 cells coated with PR8-NP or HK-NP peptides by PR8-NP or HK-NP peptide-specific CTL clones. (■) Coated with 366-374 PR8 NP (Db) peptide; (□) coated with 366-374 HK NP (Db) peptide.

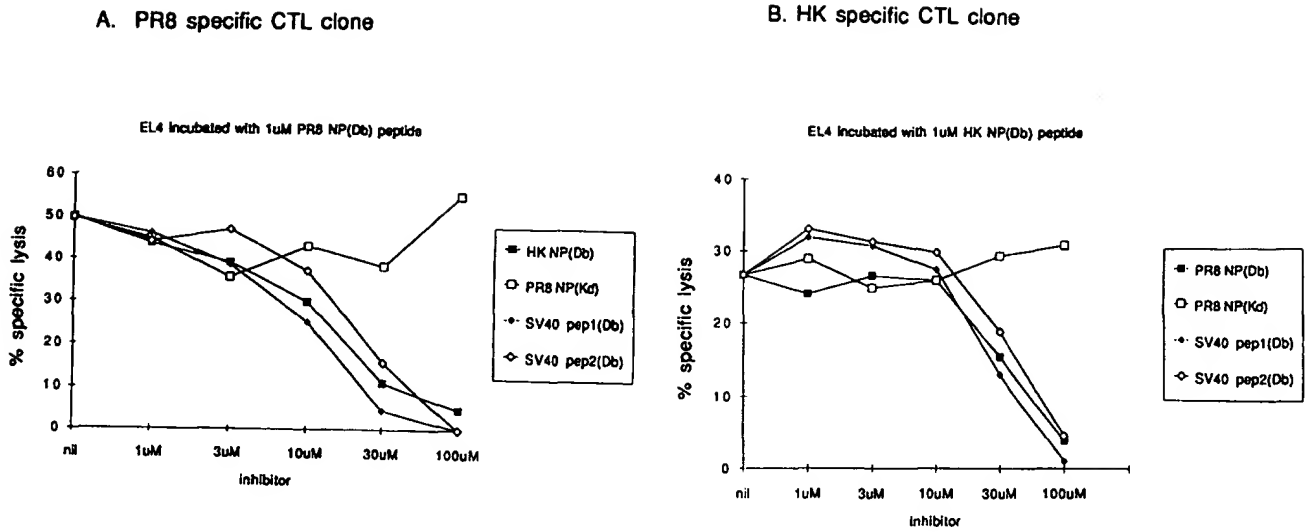

Figure 3. Inhibition of CTL activity by peptide analogs. *(A)* Specific lysis by PR8-NP specific CTL clone of EL-4 cells simultaneously incubated with 1 μM PR8-NP peptide and varying amounts of HK-NP and SV40 peptides. *(B)* Specific lysis by HK-NP-specific CTL clone of EL-4 cells simultaneously incubated with 1 μM HK-NP peptide and varying amounts of PR8-NP and SV40 peptides. The experiments were carried out at a 40:1 E/T ratio.

ments we investigated the presentation of the PR8-NP peptide expressed by the *HA* gene of the transfectant virus R10 and that of PR8 virus which contains the peptide in the NP. C57BL/6 mice were immunized with PR8 virus and splenic lymphocytes were secondarily stimulated in vitro with SC coated with PR8-NP peptide. The CTL activity was determined using PR8-NP peptide–sensitized EL-4 target cells or cells infected with PR8 or R10 viruses. The data show that, as expected, cells from nonimmunized mice, whether or not secondarily stimulated with PR8-NP peptide–coated cells, did not lyse EL-4 cells (Fig. 4, *A* and *B*). Similarly, cells from PR8 virus–immunized mice which were not restimulated in vitro with PR8-NP peptide–coated cells did not lyse EL-4 cells (Fig. 4 *C*). In contrast, cells from PR8 virus–immunized mice after secondary in vitro stimulation with PR8-NP peptide–coated SC, lysed EL-4 target cells coated with homologous peptide or infected with PR8 or R10 viruses (Fig. 4 *D*). These results show that the PR8-NP peptide is generated from the PR8 virus NP as well as from the R10 virus HA and that it is efficiently presented to CTL. More importantly, the NP peptide generated from the R10 HA was immunogenic in vivo since upon in vitro incubation with PR8-NP peptide–coated SC, cells from mice immunized with R10 virus were able to lyse EL-4 target cells infected with R10 or PR8 viruses or sensitized with PR8-NP peptide (Fig. 4 *F*). These results demonstrate that the same core peptide expressed in genes coding for different viral proteins can prime the peptide-specific CTL precursors in vivo.

The results were confirmed by experiments in which splenic lymphocytes from mice immunized with PR8, HK or R10 viruses were secondarily stimulated in vitro with PR8-NP or HK-NP peptide coated SC, and then tested for CTL activity on PR8, HK, or R10 virus infected EL-4 cells. The data depicted in Table 1 show that as expected, cells from nonimmunized mice do not exhibit CTL activity upon in vitro stimulation with PR8-NP or HK-NP peptide-coated SC. Cells from PR8 virus–immunized mice stimulated secondarily in vitro with PR8-NP–coated SC do not exhibit cytotoxic activity against HK virus–infected cells, however, they lyse target cells infected with PR8 or R10 viruses. In contrast, cells stimulated with HK-NP peptide–coated SC show a low but significant lytic capacity of EL-4 target cells infected with PR8 or R10 viruses. This activity can be attributed to cross-reactive CTL (25) that were primed in vivo and that are not eliminated by a single cycle of in vitro stimulation with peptides, the method used to generate CTL clones. Cells from mice immunized with HK virus show a significantly lower frequency of cross-reactive clones since upon in vitro stimulation with PR8-NP peptide–coated SC, low lytic activity is observed using target cells infected with PR8, HK, and R10 viruses. After in vitro stimulation with HK-NP peptide–coated SC, these cells showed significant lysis of EL-4 target cells infected with HK and R10 viruses.

It is important to note that whereas CTLs from PR8 virus–primed mice stimulated in vitro with HK-NP peptide were able to kill target cells infected with PR8 virus, those four animals primed with HK virus did not. Asymmetrical expansion of CTLs from PR8 virus–primed animals by HK-NP peptide suggest that the PR8 virus priming stimulated cross-reactive CTL or a discrete subset of CTLs exhibiting a promiscuous recognition. This may reflect the fact that PR8 virus replicates much more efficiently in mice than HK virus.

Table 1 also demonstrates that cells from animals immunized with R10 virus stimulated in vitro with PR8-NP or HK-NP peptide-coated SC display cytotoxic activity against PR8, HK, and R10 virus-infected EL-4 cells. Thus, the R10 virus can prime in vivo CTL for lysing target cells infected with both PR8 and HK virus.

These results clearly demonstrate that the R10 virus can efficiently prime both NP specific and cross-reactive CTLs

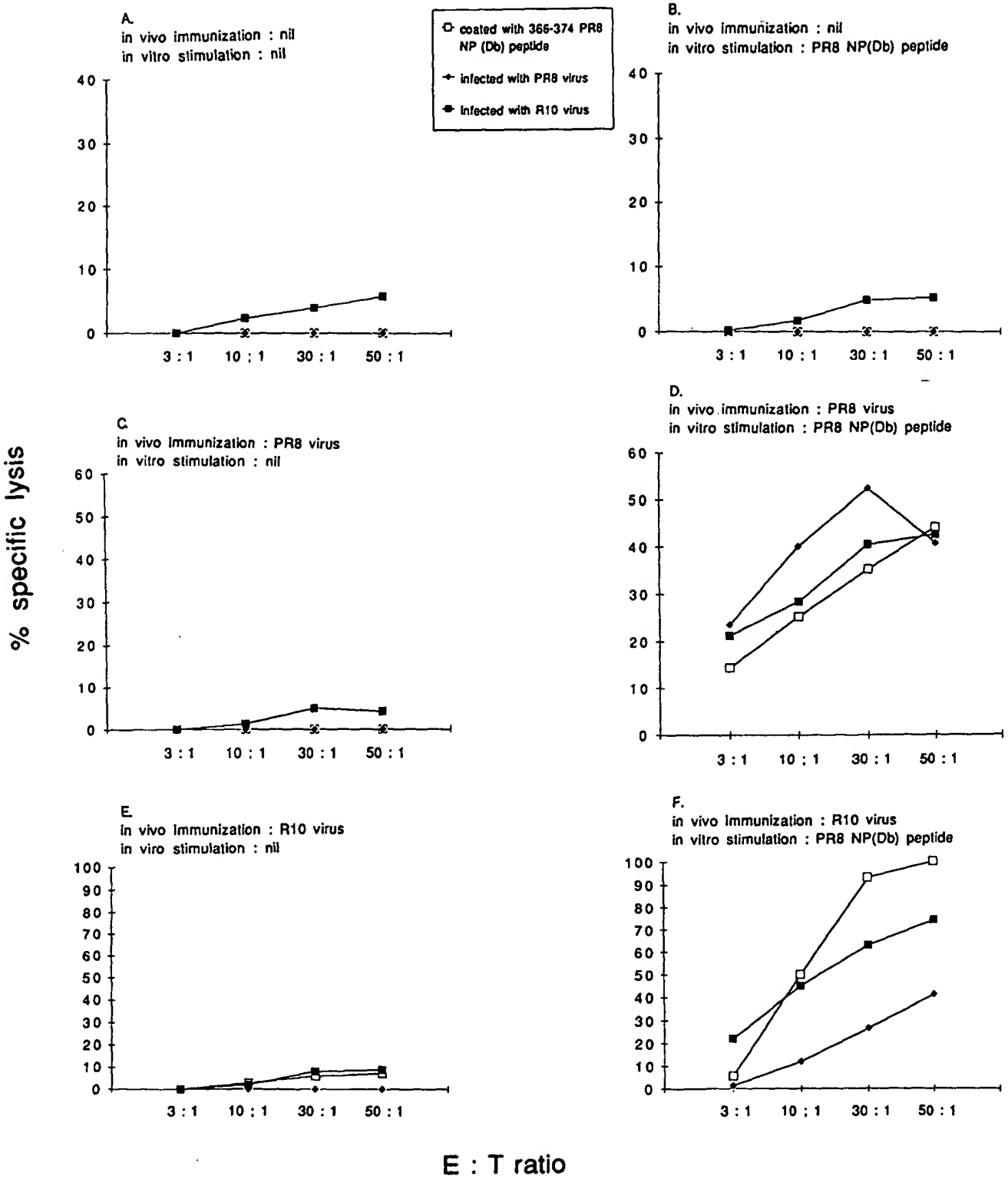

Figure 4. In vitro expansion of PR8-NP peptide-specific CTL obtained from mice that were immunized with PR8 virus or transfectant virus R10.

Table 1. *In Vivo Priming of CTL by R10 Influenza Virus Transfectant*

Mice mmunized with	Number of mice started	In vitro stimulation with spleen cells coated with	EL-4 cells infected with PR8 virus	EL-4 cells infected with HK virus	EL-4 cells infected with R10 virus
		nil	1.2 ± 1.5*	1.3 ± 1.1	0
Saline	4	PR8 NP	0.8 ± 1.2	0	0
		HK NP	0.8 ± 1.0	0	0
		nil	2.5 ± 1.8	0.3 ± 0.3	0
PR8 virus	6	PR8 NP	43.7 ± 21.5	2.2 ± 2.4	35.8 ± 10.9
		HK NP	28.6 ± 12.2	1.6 ± 2.7	37.4 ± 6.2
		nil	1.2 ± 1.0	1.0 ± 1.9	0.1 ± 0.4
NK virus	6	PR8 NP	11.2 ± 3.2	6.1 ± 7.3	14.1 ± 9.3
		HK NP	12.6 ± 4.6	37.7 ± 21.9	38.9 ± 8.0
		nil	3.7 ± 2.8	5.8 ± 5.2	0
R10 virus	6	PR8 NP	33.9 ± 16.8	43.5 ± 13.0	36.6 ± 16.0
		HK NP	18.2 ± 6.5	45.7 ± 24.0	37.2 ± 11.6

All the experiments were performed at a 30:1 E/T ratio.
NP, NP peptide
* specific cytotoxity—average ± SD.

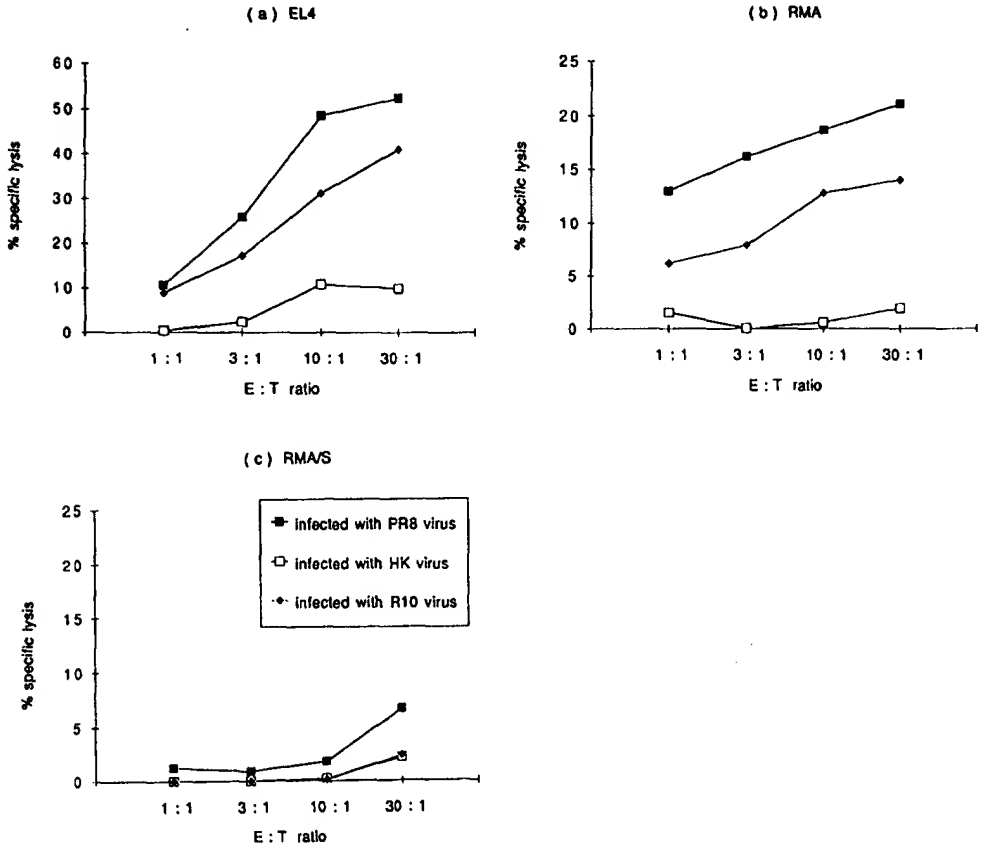

Figure 5. Comparison of lysis of EL-4, RMA, and RMA-S cells infected with PR8, HK, or R10 viruses by PR8-NP peptide-specific CTL.

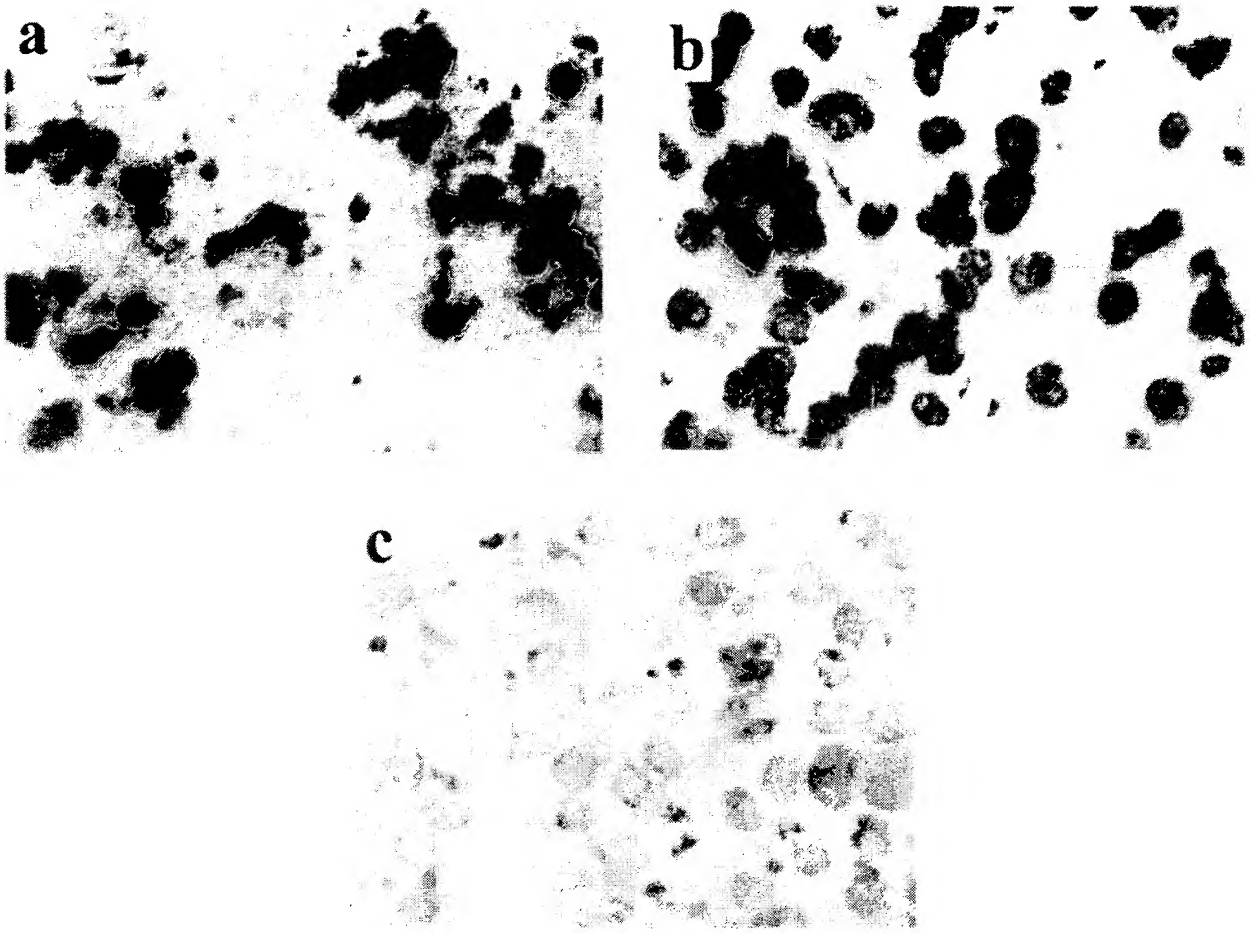

Figure 6. Expression of HA on the surface of RMA-S cell infected with PR8 and R10 viruses. RAM-S cells were incubated for 60 min with PR8 or R10 seed virus, washed, and cultured overnight. 5 × 10^4 RMA-S–infected cells were fixed for 5 min with 1% paraformaldehyde, washed, and incubated for 60 min with mAb (10 μg/ml). After washing, the cells were incubated for 45 min with peroxidase-labeled goat anti–mouse IgG (1:500 dilution), washed twice, and incubated in AEC substrate medium according to the manufacturer's instructions (Dako, Copenhagen, Denmark). (*a*) Immunostaining: virtually all cells infected with PR8 virus are stained after incubation with PY211 mAb-specific for PR8 virus HA (H1). ×200. (*b*) Immunostaining: the majority of cells infected with R10 virus are stained after incubation with 2G9 mAb specific for WSN virus HA × 200. (*c*) Immunostaining: lack of staining of cells infected with R10 virus after incubation with PY206 mAb-specific for X31 virus HA (H3). ×200.

compared with PR8 or HK viruses. It is also possible that within APCs infected with R10 virus, both PR8 and HK-NP peptides are generated and therefore CTL precursors exhibiting specificities for these peptides were expanded. These results, taken together, indicate that both peptides are generated from R10-transfectant virus. Whether residing in the chimeric HA or the native NP molecule, they are efficiently presented by D^b MHC and are able to expand precursor CTL in vivo and be recognized by CTL effectors in vitro.

Presentation by RMA-S Cells of NP Peptides Expressed by the HA and the NP Transfectant Virus R10. It has been reported that peptides derived from a signal sequence are processed by a transporter-independent pathway (26, 27). Since the PR8-NP peptide is harbored in the HA and has a signal sequence, whereas the HK-NP peptide is carried by the NP protein which is devoid of a signal sequence, it seemed possible that the HA peptide might be presented in the TAP-defective RMA-S cell line whereas the NP peptide would not. RMA-S cells have a defect in MHC class I assembly due to a defined mutation in the *TAP-2* gene (21) that prevents expression of transporter molecules (28). These transporters are believed to be involved in the translocation of peptides to the endoplasmic reticulum (ER) where they encounter MHC class I glycoproteins (29, 30).

The control experiments show that both EL-4 (Fig. 5 *a*) and RMA (Fig. 5 *b*) cells infected with PR8 or R10 viruses (and not those infected with HK virus), are lysed by PR8 NP-specific CTL. The specific lysis of PR8 virus–infected cells was somewhat higher than that of cells infected with R10 virus. This difference reflects the reduced infectivity titer of the transfectant virus (10^8 $TCID_{50}$/ml) relative to that of PR8 virus (2.8 × 10^9 tissue culture infectious dose [$TCID]_{50}$/ml). Although RMA-S cells sensitized with PR8-NP or HK-NP peptides were efficiently lysed by PR8-NP or HK-NP specific CTL (data not shown), lysis was not observed in the case of RMA-S cells infected with PR8 or R10 viruses (Fig. 5c). Lack of lysis of RMA-S target cells infected with PR8 or R10 viruses was not related to a defective production of the

HA trimer. The HAs are expressed on the surface of RMA-S cells as demonstrated by immunostaining with mAbs specific for the HA of PR8 or WSN viruses, respectively. (Fig. 6). The presence of HA of PR8 and WSN viruses on the surface of cells infected with R10 viruses was also visualized by hemadsorption (data not shown). Thus, the inability to lyse RMA-S–infected cells suggests that both peptides are processed by a transporter-dependent pathway.

Discussion

Solution at 2.4 Å resolution of the structure of the H-2D^b glycoprotein associated with the NP peptide (amino acids 366-374) of the PR8 virus has recently been reported (31). The structure reveals a hydrophobic ridge in the antigen-binding cleft of the glycoprotein, produced by the side chains of Trp73, Tyr156, and Trp147. This ridge was shown to be responsible for a bulge in the backbone of the bound peptide at P6, P7, and P8 that causes these peptide residues to be directed out of the cleft towards external solvent. Based on the high solvent-accessible surface areas of P6M, P7E, and P8T, Young et al. (31) suggested that these residues are available for contact by the TCR.

Our experimental data are in agreement with this prediction since CTL specific for the PR8-NP peptide (P7E and P8T) cannot lyse target cells coated with the HK-NP peptide (P7D and P8A) and HK-NP–specific CTL clones cannot lyse EL-4 cells coated with the PR8-NP peptide. Hence, since the identity of the side chains of the peptide residues at P7 and P8 are crucial to CTL recognition, these peptide residues must be involved in contact with the TCR. Fig. 7 shows the conformation of the PR8-NP peptide from the 2.4 Å resolution crystal structure. Superimposed on this are P7 and P8 from a model of the HK-NP peptide, obtained by replacing Glu and Thr at P7 and P8 of the PR8-NP peptide with Asp and Ala, respectively, and then performing 100 cycles of energy minimization using Insight (Biosym Technologies, CA). From this model, we predict that the differing CTL response of the two peptides is due to the fact that the HK-NP peptide has a smaller surface area available to the TCR, as a result of its having smaller side chains at P7 and P8 than the PR8-NP peptide.

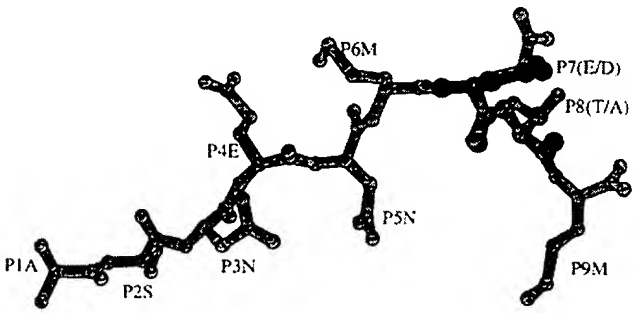

Figure 7. The 2.4 Å resolution structure of the PR-NP peptide (*gray*) and P7 and P8 of a model of the HK-NP peptide (*black*). Peptides are viewed from the side, looking from the α_2 helix toward the α_1 helix of the H-2D^b molecule. Figure produced using Molscript (55).

Our results suggest that the two NP-specific peptides have similar affinity for the MHC molecule. When 1 μM PR8-NP peptide was incubated simultaneously with varying amounts of HK-NP or of two SV40 peptides, similar levels of inhibition of target cell lysis by PR8 NP-specific CTL were observed. Comparable results were obtained when the cells were incubated with HK-NP peptide and varying amounts of PR8-NP or SV40 peptides. The conclusion that PR8-NP and HK-NP peptides have similar affinities for the D^b MHC glycoprotein was strengthened by experiments in which EL-4 cells were first incubated with a peptide for 30 min, washed, and then incubated for 2 h with peptide analogues. In this case, lysis of EL-4 cells was not inhibited by the addition of peptide analogues. This result suggests that a peptide bound to surface class I molecules cannot be easily displaced by analogues.

We sought to determine, based on these findings, whether there is competition between the PR8-NP and the HK-NP peptides when they are expressed by the HA and NP proteins of the same virus. This is important if transfectant viruses such as R10 are to be used as vaccines, since peptides specific for a single allele may compete with one another for presentation.

Our results strongly suggest that in cells infected with the transfectant virus R10 both peptides are presented efficiently. Indeed, EL-4 cells infected with R10 virus were lysed by both PR8-NP and HK-NP peptide-specific CTLs. Furthermore, immunization of mice with R10 virus primed the precursors of both PR8-NP and HK-NP peptide-specific CTLs. These results are of twofold importance. First, they show that the PR8-NP peptide expressed by the HA of R10 virus is processed like the HK-NP peptide carried by the NP protein and therefore that it appears that the location of the epitope has little influence on its presentation in this system. Our data strengthen previous observations that a peptide recognized by T cells can be generated independent of flanking regions (23, 32, 33, 34). Actually, recent data showed that an influenza virus HA epitope recognized by CTL could be moved to a different site within the HA without precluding its generation from various locations and recognition by CTLs (35). Second, lack of competition between the peptides in our system suggests that live influenza viruses may be good vectors for presenting multiple CTL epitopes useful for the immunization of outbred species.

Whereas the CTL clones generated from acutely infected mice with PR8 or HK viruses and repeatedly stimulated in vitro with NP peptide exhibited high specificity for PR8 or HK-NP peptides, the polyclonal CTL obtained from animals infected with PR8 or R10 viruses exhibited cross-reactivity. Thus, CTLs from animals primed with PR8 virus and secondarily stimulated with HK-NP peptide lysed PR8-infected cells or secondarily stimulated with PR8-NP peptide lysed HK-infected cells.

There is little information on cross-reactivity in the recognition of unrelated viral antigens. Cross-reactive recognition was reported in the case of human CTL specific for influenza matrix protein and VP4 peptide of human rotavirus (36), as well as murine CTLs specific for influenza virus NP

and PB2 peptides (37), HA and NS1 (38), and lymphocytic choriomeningitis virus (LCM), Pichinde, and vaccinia viruses (39). Furthermore, cross-reactivity was also reported at a protective level (i.e., heterosubtypic immunity) against X31 virus in tracheal and lung $CD8^+$ T cells of animals immunized with PR8 virus (40).

Several hypotheses can be entertained to explain the presence of cross-reactive CTLs in a fraction of polyclonal population obtained from mice infected with PR8 or R10 viruses. The possibility of the presence of leader sequence in the chimeric HA molecule expressing PR8-NP epitope is in agreement with the observation reported by Bacik et al. (41) showing that leader sequence enhanced the degree of cross-recognition of vesicular stomatitis virus (VSV) peptide by Sendai virus NP-specific $CD8^+$ T cells. In addition, stimulation of memory cells that cross-react at a remote level with MHC-heterologous peptide complex may be due to enhanced expression of IL-2R or adhesion molecules that render the precursors more sensitive to low affinity peptides.

It is generally accepted that the peptides derived from proteins synthesized within the cytoplasm are generated by proteasome-associated enzymes and that they are then transported by TAP transporters to the ER where they bind to class I molecules (21, 42). The lack of competition is consistent with the observation that TAP contains a recognition peptide site with broad specificity (43) and that anchor residues important for the binding to class I molecules are not important for TAP recognition and translocation (44).

It has been shown that peptides derived from signal sequences themselves are not TAP dependent, suggesting that they are produced in ER by the action of signal peptidases (26, 27). However, the majority of peptides generated from endogenous proteins without (28, 29) or with (45) signal sequences are TAP dependent. Addition of leader sequences to NH_2, but not COOH-termini of peptides which can be presented in a TAP-independent pathway, enhances the presentation in a TAP-dependent manner (41, 46). However, there are data indicating that signal sequences can bypass the requirement of TAP. Thus, Hammond et al. (47) showed that HIV-1 env protein is processed in infected cells after its cotranslation and translocation into ER via a TAP-independent pathway. Similarly, the addition of a signal sequence to an influenza virus matrix peptide can circumvent TAP deficiency in human mutant T2 cell lines. Mutant T2 cells expressing M57-68 peptide without signal sequence were not susceptible to lysis whereas those expressing the peptide with signal sequence were lysed by peptide-specific CTLs (48).

Thus, it was interesting to determine whether NP peptides expressed by the HA or NP proteins may use different processing pathways leading to their presentation on the MHC class I molecules. To this end, we have studied the lysis of R10 virus-infected RMA-S cells by PR8-NP-specific CTLs. RMA-S mutant cells have a defect in the assembly of MHC molecules (28) due to a defined mutation (21) preventing the expression of the TAP-2 gene product, a transporter involved in antigen presentation. Spies et al. (29) and later Attaya et al. (49) have shown that CTL clones specific for the NP peptide were unable to kill influenza virus RMA-S–infected cells. In contrast, there are reports showing that RMA-S cells are leaky since they are able to present VSV, Sendai, and Rauscher virus-derived peptides (50–52). Our results showed that whereas both EL-4 and RMA cells infected with PR8 or R10 viruses are lysed by PR8-NP–specific CTLs, RMA-S cells infected with the same viruses are not. Our results are in agreement with the observation of Spies et al. (29) that RMA-S mutant cells do not present the peptides derived from the influenza virus NP. More importantly, we observed that RMA-S cells do not present the PR8-NP peptide expressed by the HA gene of the R10 virus.

Failure of RMA-S cells to present the NP peptide expressed in the HA can be interpreted in two ways. First, the PR8-NP peptide is generated by the processing of the HA in the cytosolic compartment. A portion of native polypeptides with signal sequences are processed within the cytosol before translocation to ER allowing for a transporter-dependent antigen presentation. This finding is in conflict with data demonstrating that an influenza virus NP peptide located COOH-terminal to an ER insertion sequence was efficiently presented by TAP-deficient cells (48, 53). A potential reason for this discrepancy is the location of the peptide versus the leader sequence. Whether in the above mentioned experiment the peptide was located at COOH termini of the insertion sequence, in R10 virus chimeric HA, the PR8-NP peptide was located in site E, distal to signal sequence. However, it is worth noting that it was found that signal peptides can function even when located far away from NH_2 terminus of the peptide (54). Another possible explanation in the case of peptide minigene encoding signal sequence is that the peptide is dumped in ER in a preprocessed form, whereas in the case of chimeric HA, the molecule must be further processed.

Alternatively, proteins with signal sequences are processed in the ER and transporter molecules are required within the ER for the capturing of peptides by class I molecules. The latter hypothesis is supported by recent findings (44) that TAP molecules are complexed with newly assembled class I molecules. The TAP disassociates from this complex upon the binding of peptide to class I (44).

In summary, our study indicates that transfectant viruses expressing multiple immunogenic peptides presented by the same allelic MHC product can be used to expand precursor CTL in vivo and serve as targets for CTL lysis in vitro. Thus, such viruses can be used as vehicles to introduce multiple immunogenic peptides into the endogenous processing pathway. It also suggests that the processing of proteins with signaling sequences occurs in the cytoplasm and that the influenza HA and NP viral proteins use the same transporter-dependent pathway for presentation to class I MHC antigens. Our results thus indicate the feasibility of genetically engineering recombinant vaccines that express multiple epitopes to stimulate cellular immunity and that play an important role in the host defense against obligatory intracellular parasites.

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Human Peptide Transporter Deficiency

Importance of HLA-B in the Presentation of TAP-Independent EBV Antigens[1]

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Two siblings with a peptide TAP deficiency were recently described. Despite poor cell surface expression of HLA class I molecules, these patients were not unusually susceptible to viral infections. The majority of the cell surface-expressed class I molecules were HLA-B products as assessed by cytofluorometry and biochemical analysis. Analysis of two peptides eluted from the class I molecules expressed by TAP-deficient EBV B lymphoblastoid cell lines indicated that both were derived from cytosolic proteins and presented by HLA-B molecules. Peripheral $\alpha\beta$ $CD8^+$ T cells were present and their TCR repertoire was polyclonal. Most of the $\alpha\beta$ $CD8^+$ T cell clones studied (21 of 22) were nonreactive against cells expressing normal levels of the same HLA alleles as those of the TAP-deficient patients. However, it was possible to isolate one cytotoxic $CD8^+$ $\alpha\beta$ T cell clone recognizing the EBV protein LMP2 presented by HLA-B molecules on TAP-deficient cells. These observations suggest that in the TAP-deficient patients, $CD8^+$ $\alpha\beta$ T cells could mature and be recruited in immune responses to mediate HLA class I-restricted cytotoxic defense against viral infections. They also strengthen the physiologic importance of a TAP-independent processing pathway of the LMP2 protein, which was previously shown to contain several other TAP-independent epitopes. *The Journal of Immunology,* **1997, 158: 4555–4563.**

Major histocompatibility complex class I molecules are membrane-anchored proteins composed of a class I heavy chain associated with β_2-microglobulin (β_2m),[5] which present 8 to 10 amino acid peptide fragments of intracellular origin. Class I heavy chains associate with β_2m and peptides in the lumen of the endoplasmic reticulum, and these complexes migrate through the Golgi compartment to the cell surface. The peptides presented derive from the proteolytic degradation of proteins synthesized in the cell. These peptides must cross the endoplasmic reticulum membrane to associate with class I molecules and a peptide transporter, the so-called "transporter associated with antigen processing" or TAP, mediates their translocation (1). Consequently, intracellular Ags are generally not presented to cytotoxic $CD8^+$ T cells by TAP-deficient cell lines. A few in vitro examples of TAP-independent Ag presentation have nevertheless been reported, and several pathways may be involved (2–12).

Although mice defective for TAP1 protein (13) poorly express class I molecules, and therefore few $CD8^+$ T cells develop, analysis of the specificity of alloreactive $CD8^+$ T cells revealed that some were reactive against syngeneic cells expressing normal levels of MHC class I molecules (14, 15). Similar observations were reported using β_2m-deficient mice (16).

In a previous report, we described two HLA-identical patients, EMO and EFA, with a peptide transporter deficiency resulting from a homozygous stop mutation located at codon 273 of the *TAP2* gene (17, 18). Analysis of the class I molecules expressed at the membrane surface of lymphoblastoid cell lines (LCLs) raised from the patients showed these molecules to be expressed at low levels with the deficiency not affecting all class I molecules equally. Despite their HLA class I deficiency, the patients were not unusually susceptible to viral infections, although titration of antiviral Abs demonstrated that they had been infected by several common viruses (18). Ab titers suggested that in these patients humoral responses may be very important to their immunity against viruses such as herpes, measles, or mumps, while cell-mediated cytotoxic responses may ensure their defense against other viruses such as influenza or EBV (17, 18).

The aim of the present study was to characterize the class I molecules expressed by LCLs derived from the TAP2-deficient patients and to study $CD8^+$ $\alpha\beta$ T cells in these individuals. In

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[5] Abbreviations used in this paper: β_2m, β_2-microglobulin; LCL, lymphoblastoid cell line; IEF, isoelectric focusing.

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Table I. *HLA phenotypes of LCLs*[a]

Cell Line	HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ	HLA-DP
ST-EMO and ST-EFA	**A3**	**B63**[b]	**C-**[c]	**DR4**[e]	**DQ8**	***DPB1*0301***
ST-EMA and ST-EHA	**A3** A30	**B63**[b] B18	**C-**[c] Cw5	**DR4**[e] DR3	**DQ8** DQ2	***DPB1*0301/0401***
ST-EAH	A1 A30	B39 B18	C-[g] Cw5	DR7 DR3	DQ2	*DPB1*0501/0401*
SCHU	**A3**	B7	Cw7	DR15	DQ6	*DPB1*0402*
DOP-ND	A2 A33	B44 **B63**[b]	Cw4 C-[d]	DR13 DR15	DQ5 DQ6	*DPB1*0401/11012*
LUY	A2	B51	**C-**[c]	DR8	DQ7	*DPB1*0101/0401*
ZOK	A1 **A3**	B7 B58	Cw7 C-[d]	DR13 **DR4**[e]	DQ2 DQ6	*DPB1*0201/1701*
WT51	A23	B65	Cw8	DR4[f]	**DQ8**	*DPB1*02012*
BM15	A1	B49	Cw7	DR11	DQ7	***DPB1*0301***

[a] Serologic typing of HLA-A, B, -C, -DR, and -DQ alleles is given. Molecular typing of HLA-B, -C, and -DR4 was determined if necessary, while HLA-DP was typed using DNA methods. MHC molecules shared with ST-EMO are indicated in bold characters. C- indicates HLA-C blank by serology.
[b] *B*1516.*
[c] *C*1402.*
[d] C blank by serology; specificity not determined by molecular biology.
[e] *DRB1*0405.*
[f] *DRB1*0401.*

addition, the implication of $CD8^+$ αβ T cell responses in the defense against viral infections was evaluated by analyzing the reactivity of these cells toward a TAP-deficient EBV-transformed LCL derived from one of the two patients.

Materials and Methods

Reagents and media

Protein A-Sepharose, Nonidet P-40, PHA, and PMSF were obtained from Sigma Chemical Co. (St. Louis, MO) and ^{125}I-labeled and [^{35}S]methionine from Amersham (Buckinghamshire, U.K.). Synthetic peptides were provided by Neosystem (Strasbourg, France). RPMI 1640 and FCS were purchased from Life Technologies (Gaithersburg, MD) and pepstatin from Boehringer (Mannheim, Germany).

Antibodies

MAbs W6/32 (anti-class I molecules) and BM-63 (anti-β_2m) were purchased, respectively, from Valbiotech (Paris, France) and Sigma Chemical Co. MAbs 4E2, B1.23.2 (anti-HLA-B and -C), 10W070 (anti-HLA-Bw4), and GAP-A3 (anti-HLA-A3) were kindly provided by Drs. D. Charron, B. Kahn-Perles, K. Gelsthorpe, and P. Cresswell, respectively. Other mAbs employed for flow cytometry were obtained from Immunotech (Marseille, France) or Becton Dickinson (San Jose, CA). The following TCR region-specific mAbs, a generous gift from Immunotech, were used for flow cytometry: BMA031 (pan-β), E2.2E7.2 (anti-BV2S1), LE89 (anti-BV3S1), IMMU157 (anti-BV5S1), 36213 (anti-BV5S2), OT145 (anti-BV6S7*1), 3G5D15 (anti-BV7S1), 56C5.2 (anti-BV8S1/S2), FIN9 (anti-BV9S1), C21 (anti-BV11S1), S511 (anti-BV12S1), IMMU1222 (anti-BV13S1), JU74 (anti-BV13S6), CAS1.1.13 (anti-BV14S1), TAMAYA1.2 (anti-BV16S1), E17.5F3 (anti-BV17S1), BA62.6 (anti-BV18S1), ELL1.4 (anti-BV20S1), IG125 (anti-BV21S3), IMMU546 (anti-BV22S1), and HUT78#1 (anti-BV23S1), according to references given in the 1995 TCR Workshop, San Francisco, CA.

Cells

ST-EMO, ST-EHA, ST-EMA, and ST-EAH are LCLs raised from one class I deficient-patient (EMO), his father (EHA), one haploidentical brother (EMA), and an HLA-disparate brother (EAH). These cell lines have been previously described (17). BM15 (IHW9040), DOP-ND (IHW9225), LUY (IHW9070), SCHU (IHW9013), and WT51 (IHW9029) are LCLs from the XIIth International Histocompatibility Workshop cell line panel, while ZOK is an LCL raised from a local normal blood donor. HLA phenotypes of these cell lines are given in Table I. To derive T cell clones, PBLs of the TAP-deficient patient EFA were sorted with $CD8^+$ immunomagnetic beads and cultured or cloned as previously described; the cells were stimulated under polyclonal activation conditions using PHA (0.5 μg/ml), irradiated allogeneic PBLs, and LCLs in RPMI 1640 medium supplemented with 10% human serum, 1 mM L-glutamine, and rIL-2 (100 U/ml) (19, 20).

Transfection of COS cells and vectors

Transfection of COS cells was performed by the DEAE-dextran chloroquine method as described (21). In brief, 1.5×10^4 COS cells were transfected with 100 ng of an HLA-B*1516 expression vector and 100 ng of an expression vector comprising DNA coding for various EBV proteins. The HLA-B*1516 cDNA was cloned into the *EcoRI* and *XhoI* sites of pcDNA3 (Invitrogen, Leek, The Netherlands). Expression vectors containing cDNA coding for EBNA1 (pRc/CMVEBNA1), EBNA2 (pSG5 EBNA2), EBNA3a (p7CMVE3a), EBNA3b (pC7CMVE4), EBNA3c (p7CMVE6), and BRLF1 (pRc/CMV R) were kindly provided by Dr. A. Sergeant (Ecole Normale Superieure, Lyon, France). An expression vector containing a cDNA encoding LMP2 (pSG5-LMP2) was kindly provided by Dr. A. Rickinson (Cancer Research Campaign Laboratories, Department of Cancer Studies, Birmingham, U.K.). BMLF1 and BZLF1 cDNAs, cloned into pcDNA3, were derived from a cDNA library prepared with RNA from B lymphoblastoid cells (22).

T cell stimulation assay

Transfected COS cells were tested in a CTL stimulation assay 48 h after transfection. 5×10^3 cells from the T cell clone 8.24 were added to COS 48 h after transfection, and culture supernatants were harvested 6 h later and tested for TNF content by measuring culture supernatant cytotoxicity to Wehi 164 clone 13 in a colorimetric assay (23).

MHC precipitation, peptide extraction, and analysis

These procedures were conducted according to Falk et al. (24). Briefly, ST-EMO cell pellets (20 to 40 ml) were lysed in 1% (w/v) Nonidet P-40, 1 mM PMSF, and 0.02% pepstatin, and the lysates were ultracentrifuged for 60 min at 86,000 × *g*. The supernatants were immunoadsorbed onto W6/32-Sepharose beads. Peptides were acid released with 0.1% trifluoroacetic acid, and Ab molecules, HLA heavy chains, and β_2m were removed from the peptides by passage through ultrafiltration membranes (Centricon 10, Amicon GmbH, Witten, Germany). The filtrates were then separated on a reverse phase HPLC column (μRPC C2/C8, 2.1 × 100 mm, Pharmacia, Uppsala, Sweden) using a SMART system (Pharmacia). Single-peptide peaks were sequenced by Edman degradation in a pulse liquid protein sequencer 476A (Applied Biosystems, Weiterstadt, Germany).

Iodination of membrane proteins

ST-EMO cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated human serum and membrane proteins were labeled with ^{125}I using the lactoperoxidase method.

Determination of HLA-B63 subtype

RNA from the ST-EMO cell line was reverse transcribed, and the HLA-B63 cDNA was amplified by PCR. Oligonucleotides for PCR were GGGGTGATCATGCGGGTCACG-GCGCCC and GGGGTGATCAAGC TGTGAGAGACACATC; the underlined characters represent additional nucleotides introduced to facilitate further cloning experiments. The subtype was identified by digesting the amplified fragment with *KpnI* and *BsrI*,

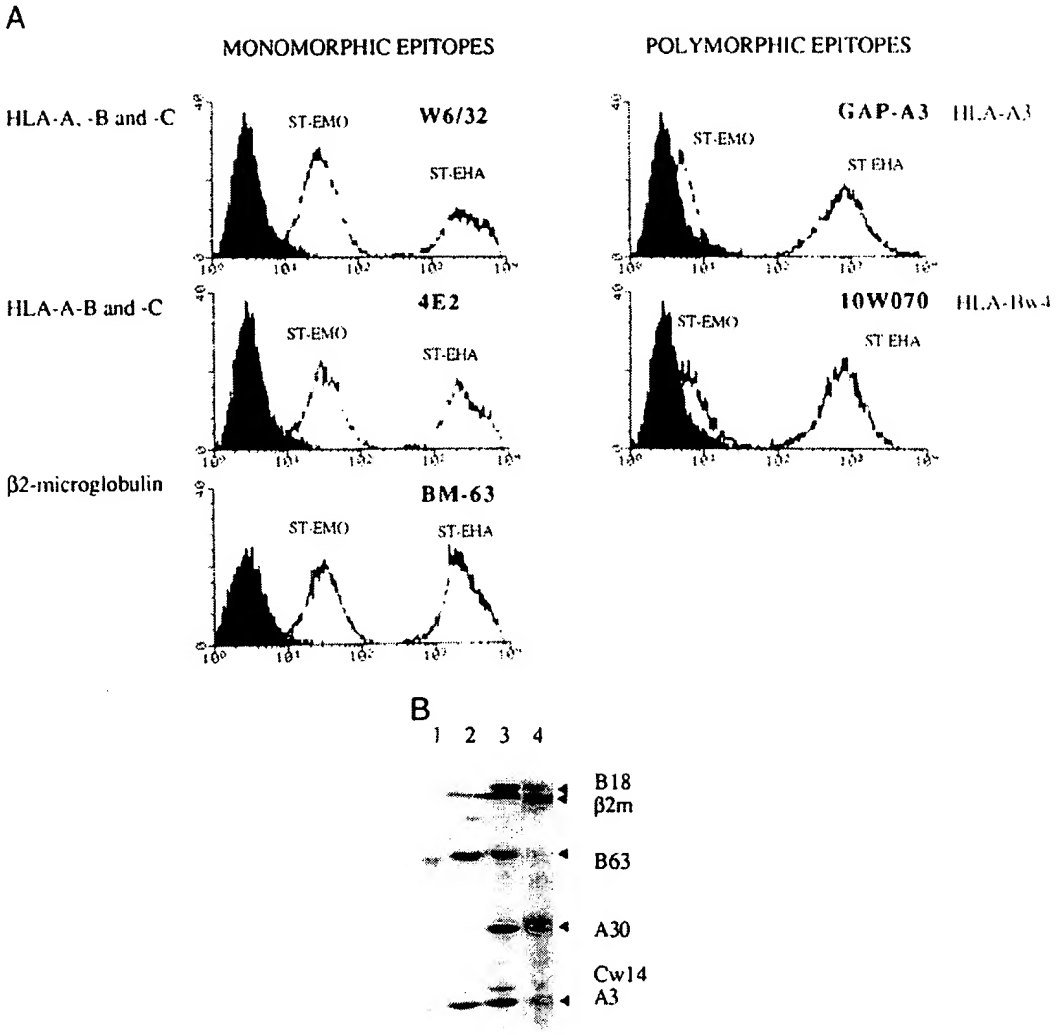


FIGURE 1. Cell surface expression of HLA class I molecules on ST-EMO cells. *A*, Flow cytometric analysis of the expression of HLA-A, -B, and -C molecules. ST-EMO (TAP2$^-$TAP2$^-$) and ST-EHA (TAP2$^+$TAP2$^-$) cells were labeled with the mAbs W6/32 (anti-HLA-A, -B, and -C), 4E2 (anti-HLA-B and -C), BM-63 (anti-β_2m), GAP-A3 (anti-HLA-A3), and 12W070 (anti-HLA-Bw4). *B*, Immunoprecipitation of HLA class I molecules. ST-EMO (*lanes 1* and *2*) and ST-EHA (*lanes 3* and *4*) cells were metabolically labeled with [^{35}S]methionine (*lanes 2* and *3*) or surface iodinated with ^{125}I by the lactoperoxidase technique (*lanes 1* and *4*). For metabolic labeling, 5×10^6 cells ST-EMO or ST-EHA were used; for cell surface iodinations, 7.5×10^6 ST-EHA cells and 7.5×10^7 cells ST-EMO cells. The cells were lysed and HLA class I molecules were immunoprecipitated with W6/32, treated with neuraminidase, and separated on IEF gels.

which allowed discrimination of the *B*1516* and *B*1517* alleles and showed EMO to be *B*1516* homozygous. Sequencing of a cDNA clone confirmed identity with the published *B*1516* sequence (25), this HLA-B isoform expressing the HLA-Bw4 epitope.

Flow cytometry

Cells were stained by two-color immunofluorescence using unconjugated anti-TCR V region mAbs revealed by FITC-conjugated goat anti-mouse IgG antiserum and phycoerythrin-conjugated anti-CD4, CD8, or pan β mAbs. Labeled cells were analyzed on a FACScan (Becton Dickinson) with LYSIS II software.

Functional assays

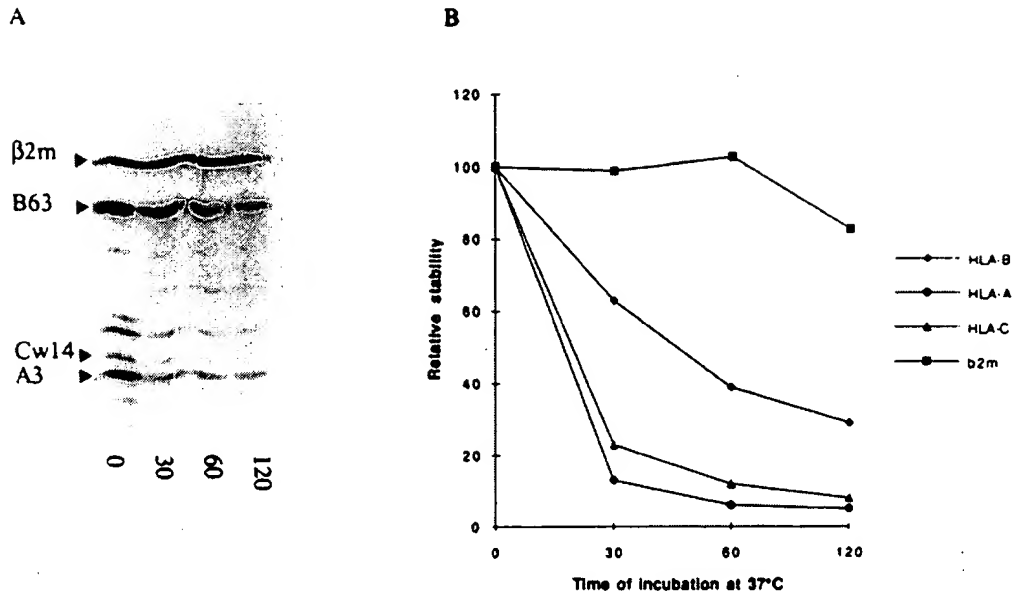
Proliferation and cytotoxicity tests were performed by standard methods (26). Briefly, the proliferative activity of responder cells was estimated by 48-h culture of 10^4 responder cells with 2.5×10^4 irradiated LCL in 100 μl of culture medium supplemented with rIL-2, followed by overnight pulsing with [^{3}H]TdR. Cytotoxic activity of T cell clones was measured by a standard 4-h ^{51}Cr release assay at different E:T ratios, and the percentage lysis was calculated as described previously (26).

Results

Residual surface MHC class I products expressed by LCL from the TAP-deficient patients are mainly HLA-B molecules

Analysis of the class I molecules present on the cell surface of LCLs derived from the patients showed that these molecules were expressed at low levels and that the deficiency did not affect all HLA class I molecules equally (17). The first aim of the present work was to analyze in more detail the cell surface expression of these molecules.

HLA class I molecules expressed on the cell surface were first characterized by flow cytometry using several mAbs directed against these molecules (Fig. 1*A*). When the expression of class I molecules on ST-EMO (TAP2$^-$TAP2$^-$) and ST-EHA



Instability of HLA class I molecules in ST-EMO cells

FIGURE 2. Instability of HLA class I molecules in ST-EMO cells. *A*, ST-EMO cells were metabolically labeled with [^{35}S]methionine and lysed, and the lysates were incubated at 37°C for different periods of time (0, 30, 60, or 120 min). HLA class I molecules were immunoprecipitated with an anti-β_2m mAb and separated on IEF gels. *B*, Densitometry of the autoradiogram. Results for each class I heavy chain and for β_2m are expressed as the percentage intensity of the corresponding signals at time 0.

(TAP2$^-$TAP2$^+$) was compared, numbers of class I molecules revealed by the mAbs W6/32 (anti-HLA class I), 4E2 (anti-HLA-B and -C), and BM-63 (anti-β_2m) were found to be reduced 100-fold on ST-EMO cells. Furthermore, HLA-A3 (*A3*0301*) and HLA-B63 (*B*1516*) molecules, detected, respectively, by GAP-A3 and anti-Bw4 mAb, appeared to be 1000-fold less strongly expressed on ST-EMO as compared with ST-EHA controls. These data implied that either HLA-C molecules were the major class I molecules expressed at the surface of the TAP-deficient cells or that the HLA-A3 and HLA-B63 molecules appearing at the cell surface had an altered conformation affecting their recognition by the anti-HLA-A3 and anti-Bw4 mAbs. To address these questions, it was necessary to characterize the class I molecules expressed at the cell surface in still further detail.

Molecules at the surface of ST-EMO (TAP2$^-$TAP2$^-$) and ST-EHA (TAP2$^-$TAP2$^+$) cells were labeled with ^{125}I, while in separate experiments intracellular proteins were metabolically labeled with [^{35}S]methionine. Class I molecules were immunoprecipitated with the mAb W6/32, treated with neuraminidase, and analyzed by isoelectric focusing (IEF). Immunoprecipitation of metabolically labeled proteins showed HLA-A3 and HLA-B63 molecules to be present at similar levels in both cell types (Fig. 1*B*, *lanes 2* and *3*). Analysis of cell surface iodinated HLA class I products immunoprecipitated with W6/32 mAb revealed that HLA-B molecules were expressed at higher level on the surface of the TAP-deficient ST-EMO cell line than HLA-A molecules (Fig. 1*B*, *lane 1*), while HLA-B molecules were less strongly expressed than HLA-A molecules at the surface of the TAP$^+$ ST-EHA cells (*lane 4*). Densitometry scanning of the autoradiograms showed that, on ST-EMO cells, the HLA-B signal was 50% higher than that of HLA-A, while on ST-EHA cells, the HLA-B signal was one-third as high as the HLA-A signal (not shown). The locations of the tyrosines in HLA-A*0301 and HLA-B*1516 molecules are identical except at positions 38 and 142, where substitutions of Tyr for Phe and His for Tyr are found in HLA-B*1516 and HLA-A*0301, respectively. Since both these substitutions are located within loops in contact with the solvent, the two molecules are most likely equally reactive to iodination, and autoradiograms should reflect levels of HLA-A and HLA-B molecules expressed at the cell surface. Thus, these experiments suggest that HLA-B*1516 molecules are less affected by the TAP deficiency than HLA-A*0301 molecules.

These properties could result from differences in the stability of the peptide-free class I molecules. To test this hypothesis, class I molecules were metabolically labeled with [^{35}S]methionine, the cells were solubilized, and the lysates were incubated for different periods of time at 37°C. Class I molecules were immunoprecipitated with an anti-β_2m mAb and separated on IEF gels (Fig. 2*A*), and the levels of class I heavy chains and β_2m were quantified by densitometry scanning of autoradiograms (Fig. 2*B*). This analysis demonstrated HLA-B*1516 heavy chain/β_2m complexes to be more stable that HLA-A*0301 and HLA-Cw*1402 heavy chain/β_2m complexes. Differences in the stability of class I molecules could therefore explain why HLA-B molecules are predominant on the surface of ST-EMO cells.

Peptides eluted from the HLA class I molecules expressed by TAP2$^-$ ST-EMO cells are presented by HLA-B molecules

To characterize the peptides presented by class I molecules, HLA class I molecules from ST-EMO cells were immunopurified with the mAb W6/32, and their peptides were eluted and separated by reverse phase chromatography. Two distinct peptides could be sequenced (Fig. 3*A*). One (peptide 1, XSTISLQM), 8 amino acids long, as expected for a peptide presented by class I molecules, comprised an unidentifiable N-terminal amino acid and a 7-amino acid sequence corresponding to human acidic calponin (NSTISLQM) (27). Glycosylation of the first amino acid could account for the lack of identification by peptide sequencing. The second (peptide 2) was at least 13 amino acids long and was found

A

PEPTIDES PURIFIED FROM HLA CLASS I MOLECULES EXTRACTED FROM ST-EMO CELLS

Pepide 1:	XSTISLQM	
	NSTISLQM	Human acidic calponin
Peptide 2:	GPASQQLSGGYGG...	
	GPASQQLSGGYGG	Human *ftp-3* gene product

B

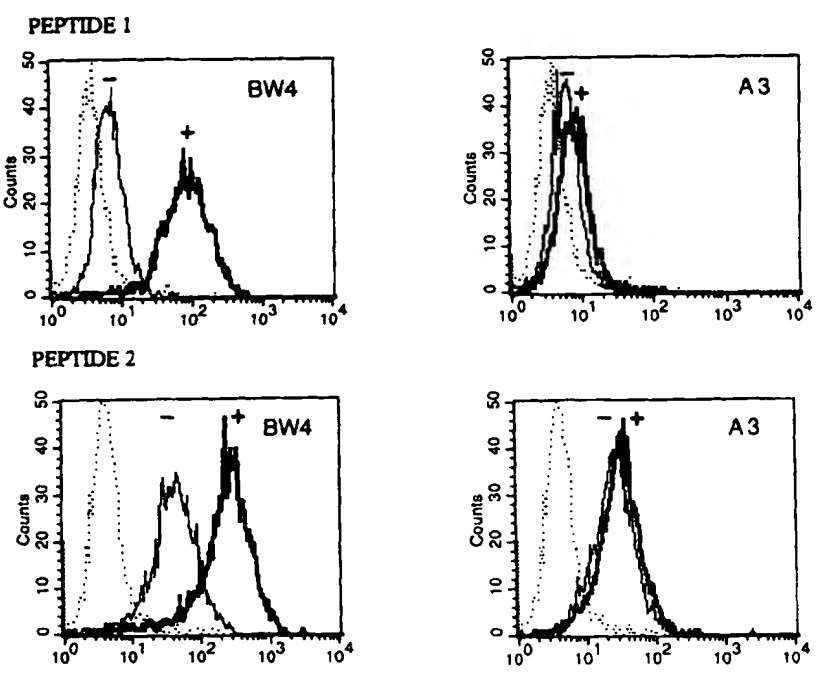


FIGURE 3. Analysis of peptides presented by HLA class I molecules on ST-EMO cells. *A*, ST-EMO cells were lysed and HLA class I molecules were immunoadsorbed onto W6/32-Sepharose. Peptides were eluted by acid treatment, separated by reverse phase chromatography, and sequenced. The amino acid sequences of two peptides could be determined, peptides 1 and 2, which show homology or identity to known protein sequences as indicated on the figure. *B*, Stabilization of HLA-B molecules by synthetic peptides 1 and 2. ST-EMO cells were incubated overnight at 27°C in RPMI supplemented with 10% FCS in the presence (+) or absence (−) of 200 μM peptide. Cells were labeled with the anti-Bw4 mAb 1OW070 or the anti-HLA-A3 mAb GAP-A3, and the stabilization of HLA class I molecules was followed by flow cytometry. Dotted lines correspond to isotypic controls. Since peptide 2 could be dissolved in water, whereas peptide 1 had to be dissolved in DMSO, controls for peptide 1 consisted of cells incubated in the presence of 5% DMSO, which explains the differences in Ab staining for cells incubated without peptide under the two sets of conditions.

in a protein encoded by the human *ftp-3* gene, located near Bruton's kinase gene (28).

The capacity of these peptides to be presented by class I molecules was confirmed by testing their ability to bind and stabilize the class I molecules expressed on ST-EMO cells. When ST-EMO cells were incubated overnight at 27°C with synthetic peptides 1 or 2 and the stabilization of class I molecules was monitored by FACS analysis with the mAb W6/32, both peptides were found to stabilize HLA class I molecules on ST-EMO cells (data not shown). In addition, using anti-HLA-A3 and anti-HLA-Bw4 mAbs, it was possible to demonstrate that HLA-B but not HLA-A molecules were stabilized by these peptides (Fig. 3*B*).

In vitro reactivity of CD8$^+$ T cell clones from TAP2-deficient patients against HLA-matched LCLs

The $\alpha\beta$ CD8$^+$ T cells maturing in MHC class I-negative mice are thought to express TCR with a high affinity for self MHC class I/peptide complexes (14–16), since part of these T cells are reactive toward syngeneic cells expressing normal levels of class I molecules on their surface. To test whether the same holds true in humans, $\alpha\beta$ CD8$^+$ T cell clones were raised from one of the TAP-deficient patients (EFA). A total of 22 randomly chosen clones were tested for their reactivity toward: 1) the fully HLA-matched ST-EMO (TAP2$^-$TAP2$^-$) cells, which express low levels of HLA-A*0301, HLA-B*1516, and HLA-C*1402 molecules; 2) ST-EMA cells derived from one brother (TAP2$^-$TAP2$^+$), which express normal levels of these HLA alleles; 3) ST-EAH (TAP2$^+$TAP2$^+$) cells derived from one brother who has not inherited the affected haplotype, which express normal levels of class I molecules; and 4) unrelated allogeneic LCLs expressing one of the HLA class I or class II alleles of the TAP-deficient patients (see HLA haplotypes in Table I). The CD8$^+$ T cell clones were heterogeneous in terms of their TCR Vα/Vβ repertoire (Table II and data not shown). Although all of the clones tested had strong lytic potential, as estimated from their lectin-dependent killing of ST-EAH, the majority (21 of 22) were unable to lyse ST-EMA (TAP2$^-$TAP2$^+$) cells. Representative results for 10 clones are given in Table II.

Table II. *Reactivity of CD8+ αβ T cell clones toward ST-EMO (TAP2−TAP2−), ST-EAH (TAP2+TAP2+), and ST-EMA (TAP2−TAP2+) LCLs*[a]

Clone	Phenotype	Cytotoxicity (% Lysis)				Proliferation (kcpm)		
		ST-EMO	ST-EMA	ST-EAH	ST-EAH + PHA	ST-EMO	ST-EMA	ST-EAH
8.7	Vβ8	0	2	0	31	0.0	0.0	0.0
8.15	Vβ13.2	0	3	3	72	0.1	0.1	0.2
8.18	Vα2	0	2	0	45	0.0	0.0	0.0
9.19	Vβ22	0	0	0	29	0.0	0.0	0.0
8.20	Vβ22	0	0	0	30	0.0	0.0	0.0
8.23	—	0	0	0	25	0.0	0.0	0.0
8.24	Vβ3	20	9	0	46	37.9	26.5	0.0
8.30	Vβ8	0	2	4	28	3.0	1.7	3.1
8.34	Vα2Vβ3	0	2	0	30	0.9	0.0	0.0
8.35	Vβ16	0	5	0	45	0.3	2.1	0.0

[a] Cytotoxic and proliferative activities of CD8+ αβ T cell clones derived from patient EFA were determined in the presence of the HLA-identical ST-EMO (TAP2−TAP2−) LCL, a heterozygous ST-EMA (TAP2−TAP2+) LCL, and an allogeneic ST-EAH (TAP2+TAP2+) LCL. The cytotoxic activity of randomly chosen T cell clones was estimated at a 10:1 E:T ratio in a 4-h ^{51}Cr release assay, while their cytolytic potential was estimated using ST-EAH in the presence of purified PHA. Proliferative activity of the clones was determined after a 48-h coculture with irradiated LCLs and expressed as (cpm of T cell clone + LCL) − ((cpm of T cells alone) + (cpm of LCL alone)). The proliferation of T cell clones in the absence of LCLs was less than 500 cpm (data not shown). TCR phenotypes of the clones were deduced by flow cytometry using TCR Vα- and Vβ-specific mAbs, but none of the available mAbs stained clone 8.23.

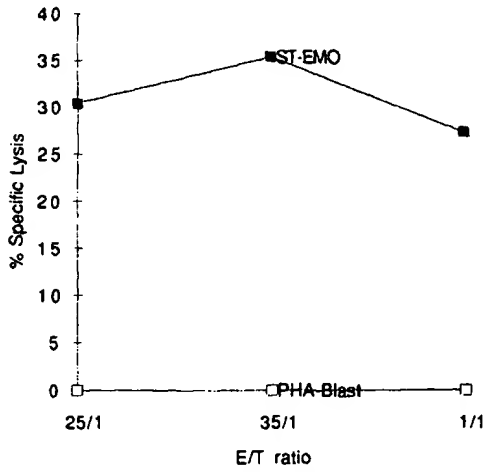


FIGURE 4. Cytotoxicity of the CD8+ αβ T cell clone 8.24 to EBV-transformed B cells. Cytotoxicity of the clone 8.24 to ST-EMO cells and to autologous EBV-free PHA T cell blasts was tested in a 4-h ^{51}Cr release assay, with results expressed as the percentage of specific target lysis.

Interestingly, one CD8+ T cell clone (8.24) killed ST-EMO (TAP2−TAP2−) and ST-EMA (TAP2−/TAP2+) cells but not the unrelated ST-EAH (TAP2+/TAP2+) cells (Table II) and, likewise, strongly proliferated when cocultured with ST-EMO or ST-EMA but not in the presence of ST-EAH cells. The other 21 clones, which were not cytotoxic to ST-EMO cells, did not proliferate under these conditions. These data suggested the specific recognition of HLA alleles by this clone, presumably of class I molecules given its CD8+ phenotype. Furthermore, this clone killed EBV-transformed ST-EMO cells but not EBV-negative autologous PHA blasts, strongly suggesting its recognition of an EBV Ag (Fig. 4). The clone turned out quite unexpectedly to be more effective against TAP2−TAP2− ST-EMO cells than against TAP2−TAP2+ ST-EMA cells (Fig. 5*A*). These results prompted us to further analyze the HLA allele specificity of this clone by testing its reactivity toward a panel of LCLs. As shown in Figure 5, clone 8.24 displayed cytotoxic and proliferative activity in the presence of DOP-ND, which shares the HLA-B63 (*B*1516*) allele with ST-EMO, but not in the presence of other LCLs sharing HLA-A, -C, -DR, -DP, or -DQ alleles with ST-EMO, suggesting its restriction by HLA-B. This hypothesis was supported by the results of blocking studies with anti-class I- or -class II-specific mAbs. Indeed, cytolysis of ST-EMO and DOP-ND cells was strongly inhibited by the anti-HLA-B/-C mAb B1.23.2 (Fig. 6) and by the anti-HLA class I monomorphic mAb W6/32 (data not shown), but was unaffected by anti-HLA-DR-, -DP-, or -DQ-specific mAbs (data not shown). A nonspecific inhibitory activity of B1.23.2 and W6/32 was ruled out by demonstrating their lack of effect on the HLA-unrestricted cytolysis of Daudi cells by a γδ T cell clone (Fig. 6 and data not shown).

Recognition of LMP2 by the T cell clone 8.24

In an attempt to identify the putative EBV Ag recognized by 8.24 cells, we evaluated its reactivity toward a large set of EBV proteins by transient COS cell transfection assay (21, 23). 8.24 cells were incubated for 48 h in the presence of COS cells cotransfected with expression vectors coding for HLA-B*1516 and for various latent or lytic EBV proteins. The amounts of TNF-α released in the culture supernatants was then quantified. Barely detectable amounts of TNF-α were produced by 8.24 cells incubated with COS cells transfected with the HLAB*1516 expression vector alone. In contrast, COS cells cotransfected with HLA-B*1516 and LMP2 DNAs stimulated 8.24 cells to produce 25 pg/ml of TNF-α. All other EBV Ags failed to induce production of TNF-α by this clone (Table III).

Discussion

In a previous study relating to the characterization of two siblings with an HLA class I deficiency resulting from a peptide transporter defect (17, 18), flow cytometry showed the cell surface expression of class I molecules to be reduced 100-fold. However, contrary to observations in class I-negative transgenic mice, this reduction in class I expression only partially blocked the development of CD8+ T cells. These individuals were, moreover, apparently healthy in early childhood. Pulmonary bacterial infections only started to appear with increasing age, while the presence of Abs against several common viruses demonstrated their ability to fight viral infections without experiencing any directly related abnormal pathology (18). Such observations raised questions with regard to the likelihood of the presentation of viral Ags by a peptide transporter-independent pathway, as has been reported in several in vitro models.

To determine whether TAP-independent Ag presentation is involved in the immune responses of these TAP-deficient patients, it

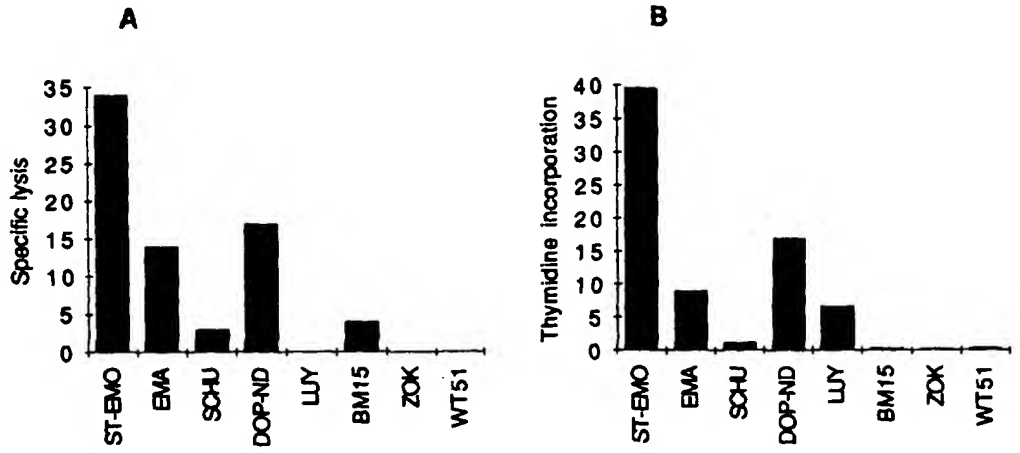


FIGURE 5. Reactivity of the T cell clone 8.24 toward ST-EMO (TAP2$^-$TAP2$^-$) cells, ST-EMA (TAP2$^+$TAP2$^-$) cells, and LCLs sharing one HLA gene with the ST-EMO cell line (Table II). *A*, Cytotoxicity was evaluated as described in Figure 4. *B*, Proliferation was estimated after a 2-day culture of 8.24 T cells with irradiated stimulator cells and expressed as (cpm of T cells + LCL) − ((cpm of T cells alone) + (cpm of LCL alone)). In these experiments, the E:T ratio was 5:1.

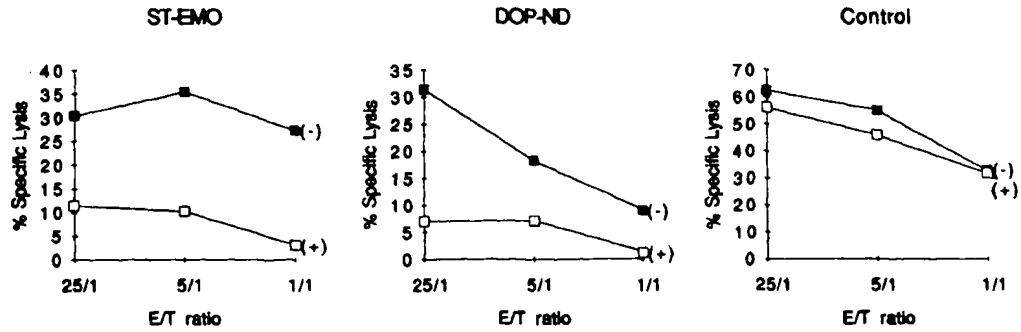


FIGURE 6. Inhibition of the cytotoxicity of the T cell clone 8.24 by an anti-HLA-B and HLA-C mAb. HLA-B-restricted cytotoxicity of the clone 8.24 was assayed using HLA-B*1516$^+$ ST-EMO and DOP-ND cells in the presence (+) or absence (−) of the anti-HLA-B and -C mAb B1.23.2. Control: absence of a nonspecific inhibitory effect was checked in cytotoxicity experiments using a γδ T cell clone and Daudi cells.

Table III. *TNF-α secretion of 8.24 T cell clone stimulated by COS cells cotransfected with HLA-B*1516 cDNA and various EBV cDNAs or HLA-B*1516 cDNA alone*

EBV Ag	TNF-α Release (pg/ml)
None	0.2
LMP2	25.9
EBNA1	0.2
EBNA2	0.2
EBNA3A	0.3
EBNA3B	0.2
EBNA3C	0.2
BRLF1	0.3
BMLF1	0.2
BZLF12	0.2

was first necessary to characterize the HLA class I molecules expressed at the cell surface. When cell surface molecules on ST-EMO (TAP2$^-$TAP2$^-$) and ST-EHA (TAP2$^-$TAP2$^+$) cells were iodinated and immunoprecipitated with the mAb W6/32, levels of HLA class I molecules were found to be strongly reduced on the surface of TAP-deficient cells, in agreement with the results of flow cytometry. The ratio of HLA-B to HLA-A molecules expressed on ST-EMO cells was 1.5, while it was 0.3 on the TAP-positive ST-EHA cells. Flow cytometry experiments using the anti-class I mAb W6/32 or anti-HLA-B and -C mAb B1.23.2 showed the cell surface expression of HLA class I molecules to be reduced 100-fold; this reduction was approximately 1000-fold when using mAbs of narrow specificity (anti-HLA-A3 or anti-HLA-Bw4). These observations suggest that 1) most of the class I molecules adopt a conformation different from that at the surface of normal cells, and 2) cell surface expression of the various HLA class I isotypes is differently affected by the peptide transporter defect. In these patients, most of the newly synthesized class I heavy chains remain unsialylated, and most HLA class I molecules, therefore, remain peptide free (17). The present results indicate that HLA-B*1516 heavy chain/β_2m complexes are apparently more stable than HLA-A and -C molecules expressed in ST-EMO cells. Such differences in stability could explain why HLA-B molecules are "predominant" on the plasma membrane of ST-EMO cells. This is, nevertheless, likely to result from biochemical properties specific to the HLA isoforms expressed by these cells and may not reflect a general feature of all HLA-A and -B products. HLA-B complexes are barely detectable at the plasma membrane of TAP-deficient T2 cells, with most of the MHC class I Ags corresponding to HLA-A2 molecules (29) that present Ags derived from signal peptides (2, 5).

Peptide presentation by the class I molecules on ST-EMO cells was studied in a second step. Despite the difficulty of characterizing such peptides due to the low levels of expression of class I

molecules on the TAP-deficient cells, two peptides presented by HLA-B molecules could be sequenced. One, which had the expected length for presentation by HLA class I molecules, is also found in the sequence of human acidic calponin, a protein of unknown function probably associated with the cytoskeleton. Human acidic calponin is expressed in many tissues, and reverse transcriptase-PCR experiments demonstrated its expression in ST-EMO cells (data not shown). A second longer peptide was presumably derived from the human *ftp-3* gene located in the region of Bruton's kinase gene. This gene is expressed predominantly in B and T cell lines and encodes a protein belonging to a family of RNA-binding proteins (28). Presentation of the two peptides could be explained if TAP1 homodimers retain weak peptide transporter activity, as has been suggested by Ag presentation experiments using TAP2-deficient murine cell lines (4).

In class I-negative mice, $CD8^+$ $\alpha\beta$ T cells are cytotoxic to syngeneic cells expressing normal levels of class I molecules, a property that has been explained by the requirement during positive selection for high affinity interactions between class I molecules and TCRs to compensate for the low avidity of T cell/target cell interactions. However, this property is not observed in T cell lines raised from TAP-deficient mice immunized with certain MHC class I-restricted peptides (30). Hence, it was of interest to test the reactivity of $CD8^+$ $\alpha\beta$ T cells from one of our patients toward TAP-positive haploidentical cells. A total of 22 $CD8^+$ $\alpha\beta$ T clones raised from EFA lymphocytes under polyclonal conditions of stimulation were tested in the presence of ST-EMO ($TAP2^-TAP2^-$) cells and of ST-EMA ($TAP2^+TAP2^-$) cells expressing normal levels of the HLA-A, -B, and -C alleles of the TAP-deficient patients. The repertoire of the Vα and Vβ regions of the TCR was heterogeneous, suggesting that the TCR repertoire in TAP-deficient patients was diverse, as in the case in TAP-deficient transgenic mice (30). Preliminary analyses by flow cytometry of the TCR repertoire of $CD8^+$ and $CD4^+$ T cells from EFA have indicated the usage frequencies of the different Vα and Vβ region to be similar in both of the T cell populations (data not shown). It has been described that in HLA class II-deficient patients the repertoire of $CD4^+$ T cells is likewise highly polyclonal (31, 32). However, bias exists in the fine structure of the TCR (33), and we are currently performing an extensive molecular analysis of the TCR repertoire of the T cells from our HLA class I-deficient patients.

Among the 22 $CD8^+$ $\alpha\beta$ T cell clones, 21 showed no cytotoxic or proliferative activity in the presence of ST-EMA ($TAP2^+TAP2^-$) cells. Hence, these T cells were not reactive toward a cell line expressing normal levels of class I molecules. This property may result from our method of raising the $CD8^+$ T cell clones, since in TAP-deficient mice alloreactive T cell clones and peptide-specific T cell lines do not display the same pattern of reactivity toward TAP^+ syngeneic cells. Therefore, at least in these class I-deficient animals, interactions between TCR and class I molecules might involve different parts of the TCR and class I molecules and thus different biophysical interactions, depending on whether these TCR are involved in allogeneic recognition or in the recognition of a particular peptide presented by autologous MHC class I molecules. This hypothesis is supported by a recent publication describing the peptide specificity of an allogeneic T cell clone, which likewise recognizes a specific peptide presented by autologous class I molecules (34). If correct, one would predict that memory T cells in these patients, which recognize TAP-independent peptides, would be poorly reactive against HLA-identical TAP^+ cells, while only the fraction of the T cells that are potentially allogeneic would be cytotoxic against HLA-identical TAP^+ cells. Alternatively, it is possible that most of our $CD8^+$ T cell clones are in fact restricted by molecules distinct from classical MHC class I products and therefore may not have been selected on the basis of their ability to interact with HLA-A, -B, or -C molecules.

One clone was cytotoxic and proliferated in the presence of ST-EMO ($TAP2^-TAP2^-$) cells. This T cell clone was found to be restricted by HLA-B*1516 and to recognize the LMP2 EBV protein. Significantly, this EBV protein was previously shown to be presented in a TAP-independent pathway to HLA-A2-, -A11-, -A23-restricted T cell clones (11, 12).

In view of the remarkable result that the anti-EBV T cell clone was restricted by HLA-B, the predominant, although still poorly expressed, class I molecule on the TAP-deficient cells, we also tested HLA-A3-restricted cytotoxic T cell lines and clones raised from normal donors specific for EBV or influenza Ags (matrix and nucleoprotein). These cell lines were unable to lyse ST-EMO cells or virus-infected ST-EMO cells (data not shown). These differences in TAP dependency may result from the subcellular localization of the Ags and their biochemical properties. Few examples of well-defined TAP-independent Ags, presented either by HLA-A or HLA-B molecules, have been described (10–12). These Ags are derived from the LMP2 and Env protein of EBV and HIV, respectively. As previously suggested, the TAP-independent processing of LMP2 may be explained by the unusual topology of this protein, which contains 12 membrane-spanning regions. Indeed, the TAP-independent antigenic peptides identified thus far are localized in two transmembrane domains and in a cytosolic fragment adjacent to one of these transmembrane domains (11, 12). It will be interesting to precisely define the B*1516-restricted epitope herein evidenced and determine whether its TAP independency is primarily due to its localization within the protein or to its hydrophobic character. The mechanisms of internalization of the peptides derived from the cytosolic and transmembrane domain of LMP2 remain to be elucidated. Passive transfer may be sufficient; alternatively, other proteins involved in the transport of peptides, such as heat shock proteins, may play a role. It should be noted that 1) several heat shock proteins appear to bind peptides and allow their presentation (35), and 2) some but not all cytosolic peptides expressed in the cytosol using minigenes can be presented in a TAP-independent pathway (9). With regard to Env, the TAP-independent epitopes are part of the ecto-domain, and become exclusively TAP-dependent when they are expressed in a truncated cytosolic soluble form (10). It has been proposed that degradation of misfolded protein by postendoplasmic reticulum and nonlysosomal proteases, or amino peptidase in endoplasmic reticulum may be involved in this TAP-independent presentation (10).

Other pathways can be involved in the TAP2-deficient patients. The hsp73 protein was implicated in the presentation of truncated, and probably misfolded, SV40 large T Ag in a pathway involving endosomal compartments (3). Such compartments were also implicated in the presentation of a Sendai virus Ag (11). Ag presentation assays have shown that the murine TAP1 subunit displays residual transporter activity (4). However, this observation is not confirmed by biochemical studies with human TAP1 protein (36). These differences may be explained by differences between human and murine TAP1 proteins or between the assays. Alternatively, since TAP1 protein is known to bind β_2m/heavy chain complexes in endoplasmic reticulum, this protein may help in the loading of HLA class I complexes with peptides delivered by alternative pathways.

The TAP dependency of the matrix and nucleocapsid protein of influenza virus may lie in their cytosolic and nuclear localization. It should be noted that T cell clones used in the assays were raised from normal individuals. In these individuals, due to the subcellular localization of the two proteins, immunodominant peptides derived from these proteins may be predominantly TAP dependent

because they are more efficiently transported than TAP-independent peptides. Complementary experiments will determine whether TAP-independent Ags derived from such proteins can be involved in the immune response of the TAP-deficient patients and which pathways are used for this presentation.

Low titers of anti-EBV Abs have been observed in both patients, although neither has suffered from the so-called chronic fatigue syndrome, which is often observed after EBV infection in patients with diminished NK activity (37). This points to a capacity for cell-mediated cytotoxic responses against EBV (18). Our in vitro data further support this conclusion and demonstrate that cytotoxic anti-EBV responses mediated by $CD8^+$ $\alpha\beta$ T cells may occur in TAP-deficient humans. The patients have relatively low numbers of $CD8^+$ $\alpha\beta$ T cells (17), although these numbers seem to increase significantly with the progression of chronic lung inflammation (data not shown). Our results suggest that these cells may mediate HLA class I-restricted TAP-independent antiviral immune responses, explaining why these patients do not suffer from abnormal viral infections.

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Antigen Processing and Presentation

TAP-Independent Delivery of Antigenic Peptides to the Endoplasmic Reticulum: Therapeutic Potential and Insights into TAP-Dependent Antigen Processing

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Summary: We have taken several approaches to investigate the capacity of the secretory pathway to liberate major histocompatibility complex (MHC) class I-restricted antigenic peptides from precursor polypeptides. Cells lacking the peptide transporter (TAP) are unable to deliver peptides from cytosolic antigens to class I molecules. TAP can be bypassed by targeting peptides directly to the endoplasmic reticulum (ER) using NH_2-terminal signal sequences. This results in the generation of enormous numbers of MHC class I complexes (50,000 peptides/cell), and recombinant vaccinia viruses expressing such peptides are highly immunogenic. In contrast to signal sequence-targeted peptides, peptides are liberated very inefficiently from internal locations in ER-targeted full-length proteins, indicating that the secretory pathway has a limited capacity for generating antigenic peptides from most polypeptide contexts. We have, however, identified a location in proteins from which peptides can be liberated in numerous contexts in the secretory pathway. Placing a number of different peptides at the COOH termini of a secreted protein and two proteins with type II membrane anchors resulted in their TAP-independent presentation. These findings demonstrate that the secretory compartment possesses proteases able to liberate COOH-terminal antigenic peptides from virtually any context, entirely consistent with a role for these proteases in the processing of TAP-transported antigenic peptide precursors. **Key Words:** Antigen processing—Cytotoxic T lymphocytes—Recombinant viruses—Vaccine.

Major histocompatibility complex (MHC) class I molecules function to present foreign and self peptides to CD8 T lymphocytes (T_{CD8+}). Successes in treating and preventing experimental animal tumors with T_{CD8+} have generated a great deal of excitement regarding the possible clinical applications of T_{CD8+}-based therapies. Pioneering work from a number of laboratories suggests that this approach has great potential in treating human malignancies.

Recent years have witnessed enormous strides in our understanding of the molecular and cell biological bases for T_{CD8+} recognition. In the last year, an important mile-

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stone was passed with the publication of the crystal structures of T cell receptor (TCR)-class I complexes (1,2). This remarkable accomplishment was the culmination of a series of discoveries stemming from the initial observation of MHC restriction by Zinkernagel and Doherty in 1973 (3). How fitting that the atomic definition of MHC restriction should appear in the year that the importance of Zinkernagel and Doherty's contribution to medicine and physiology was recognized by the Nobel committee!

The basis for MHC restriction is the interaction of the TCR with a large region of the MHC class I molecule consisting of the α helices derived from α1α2 domains of the class I heavy chain and residues from a small peptide bound in between the helices (2,4). Class I binding peptides are generally between 8 and 11 residues in length (5). More than 90% of these peptides derive from a cytosolic source, inasmuch as they are dependent on the expression of a transporter (TAP) that functions to deliver peptides to newly synthesized class I molecules in the endoplasmic reticulum (ER) (6,7). TAP efficiently transports peptides of between 8 and 16 residues in length (8). The smaller size is in good agreement with the minimal size recovered from class I molecules (6). There is a discrepancy, however, at the other end of the scale, since peptides >11 residues are infrequently recovered from class I molecules. This suggests that a fraction of peptides produced in the cytosol are longer than necessary for optimal class I binding and are trimmed in the ER following TAP transport.

Based on the TAP dependence of peptide production, it is clear that cells have only a limited capacity to produce peptides with high affinity for class I molecules from proteins exported into the secretory pathway. This is consistent with observations that most antigenic peptides are produced inefficiently, if at all physiologically, from full-length proteins targeted to the ER of TAP-deficient cells (9). There are two potential reasons for this TAP dependence. First, and most simply, TAP may be needed only to provide peptides. Second, class I molecules physically associate with TAP and at least two other proteins: an ER chaperone (calnexin or calreticulin) and an MHC gene product termed tapasin (10,11). TAP could therefore also be required for loading peptides onto class I molecules in the ER. That the first possibility is at least partially correct was first demonstrated by Anderson et al. (12), who showed that peptides delivered to the ER of TAP-deficient cells by the NH_2-terminal signal sequence of an adenovirus protein were efficiently presented to T_{CD8+}. We extended these findings to other signal sequences and multiple peptides (13). Most relevant for the present purposes, we demonstrated that recombinant vaccinia viruses (rVVs) expressing ER-targeted peptides are often more immunogenic than full-length proteins (14).

QUANTITATION OF PEPTIDE-MHC COMPLEXES TO EXPLAIN THE ENHANCED IMMUNOGENICITY OF rVVs EXPRESSING ER-TARGETED PEPTIDES

Previously we showed that cytosolic and ER-targeted minigenes are more efficiently generated than peptides from full-length proteins, as determined by their appearance on the cell surface more quickly following rVV infection (15). This provides only the roughest quantitative measure of their generation, however. To better understand the enhanced immunogenicity of ER-targeted peptides, we quantitated the amount of class I-associated peptides generated from influenza virus nucleoprotein. We studied the immunodominant peptides recognized by nucleoprotein (NP)-specific T_{CD8+} from Balb/C ($H\text{-}2^d$) and CBA ($H\text{-}2^k$) mice corresponding, respectively, to $NP_{147\text{-}155}$ (K^d-restricted) and $NP_{50\text{-}57}$ (K^k-restricted). Peptides were quantitated in high performance liquid chromatography (HPLC) fractions recovered from low molecular weight material present in acid extracts as originally described by Rammensee and colleagues (5). The amounts of peptides recovered per cell were calculated by the ability of HPLC fractions to sensitize target cells for lysis by appropriate T_{CD8+} lines. Synthetic peptides corresponding to naturally processed peptides were used to generate standard curves. The efficiency of peptide recovery was estimated by doping control extracts with known quantities of synthetic peptide.

This experiment revealed a number of unexpected features of antigen processing. First, following 6-h infection of L-K^d cells (L929 cells expressing K^d from a transfected gene) with an rVV expressing full-length NP, nearly 2,000 copies of $NP_{50\text{-}57}$ were recovered from cells. The same cells yielded only 30 copies of the $NP_{147\text{-}155}$ peptide. Amazingly, despite the very low efficiency of $NP_{147\text{-}155}$ generation from NP, this determinant still manages to suppress responses to two immunorecessive K^d-restricted NP determinants (4). Second, expressing either of the determinants as ER-targeted minigenes results in the generation of 50,000 peptides/cell. Thus, expressing the peptides as ER-targeted peptides increases the efficiency of presentation by up to nearly 2,000-fold.

This finding was independently confirmed by examining the ability of ER-targeted peptides to enhance expression of rVV-encoded mouse class I molecules in TAP-deficient cells. At 37°C, surface expression of such class I molecules is quite low due largely to the thermal instability of the class I molecules once they reach the

cell surface lacking a high-affinity ligand in their binding groove. When co-expressed with ER-targeted peptides, however, class I molecules are stably expressed and are easily detected using monoclonal antibodies specific for conformed class I molecules.

These experiments demonstrate that ER-targeted peptides induce the expression of superphysiological levels of class I-peptide complexes and that this expression occurs independently of TAP expression. This provides a ready explanation for the enhanced immunogenicity of rVVs expressing such peptides. Indeed, with such massive expression, it is possible that nonprofessional antigen presenting cells (APCs) induce primary T_{CD8+} responses. We are currently attempting to determine the identity of APCs active in stimulating primary T_{CD8+} responses following infection with rVVs. It is possible that the identity of cells varies depending on whether the rVV expresses full-length proteins versus ER-targeted peptides. In the former case, low levels of peptide-class I complexes may dictate that presentation is performed only by cells that express co-stimulatory molecules (B-7 and the like). Moreover, the expression of stable full-length antigens may enable representation of cell debris by professional APCs [originally discovered in "cross-priming" experiments (16)]. Such cross-priming is probably impossible with minigene products expressing rVVs, since the peptides are rapidly degraded in the absence of class I ligands.

It is possible the immunogenicity of rVVs expressing ER-targeted peptides will be enhanced by co-expression of the appropriate class I molecules. This follows from several considerations. First, APCs may not express saturating amounts of class I molecules. Second, VV infection is associated with inhibition of MHC class I expression, probably simply as a result of viral inhibition of host gene expression.

In any event, the practical message for immunotherapy of tumors with defined antigenic peptides is that immunization with viral or naked DNA vectors that express ER-targeted peptides (possibly with class I molecules) may be the optimal method for inducing T_{CD8+} responses. This approach may bypass the requirement for presentation by professional APCs or, if such APCs can be infected by the vector used, result in expression of high numbers of peptide MHC complexes.

EXPLORING THE LIBERATION OF T_{CD8+} DETERMINANTS FROM EXTENDED ER-TARGETED POLYPEPTIDES

In previous work we showed that the ER preferentially liberates peptides from the COOH terminus from "tandem" peptides directed to the ER of TAP-deficient cells, destroying the NH_2-terminal peptide in the process (17). We first observed this preference for COOH-terminal peptides in a prior study where we found that signal sequences had to be located at the NH_2 terminus to enable TAP-independent presentation, but the significance of this finding regarding the criticality of the COOH-terminal position for peptide liberation in the ER eluded us (13).

To determine whether COOH-terminal peptides could be liberated from longer polypeptides, we generated rVVs that express an ER-targeted form of NP (termed SNP) with COOH-terminal extensions corresponding to five distinct peptides: NP_{50-57}, $NP_{147-155}$, $NP_{366-374}$ (D^b-restricted), $OVA_{257-264}$ (immunodominant K^b-restricted peptide from ovalbumin), and N_{52-59} (immunodominant K^b-restricted peptide from vesicular stomatitis virus nucleocapsid protein). Two control rVVs were produced, in which peptides were appended to the COOH terminus of cytosolic NP. Proteins were expressed in T2 cells using rVV as a vector. T2 cells lack a mega-base pair region of the MHC that contains the *TAP1 and TAP2* genes (18). Study of the COOH terminal-reiterated influenza peptides was made possible by the inability of T2 cells to liberate the peptides from their internal locations in SNP.

In contrast to the internally located NP peptides, T2 cells were able to present, in a TAP-independent manner, each of the NP peptides when expressed at the COOH terminus of SNP. This cannot be attributed to peptide-induced alterations in SNP structure resulting in the enhanced liberation of the corresponding internal peptide, since the enhancing effect of each extension on presentation did not extend to the other two peptides expressed only in the internal location. That the COOH-terminal extension itself is processed from SNP was definitively shown by the presentation of $OVA_{257-264}$ and N_{52-59}, which were present only at the COOH terminus. For NP_{50-57}- and $Ova_{257-264}$-containing proteins, targeting of the protein to the ER was directly shown to be essential for TAP-independent presentation, since cytosolic versions of the proteins with the COOH-terminal peptides were unable to sensitize cells for lysis.

We next examined whether the $NP_{147-155}$ could be liberated from the COOH terminus of membrane-bound proteins. We chose two proteins with type II membrane anchors, Jaw1 and CD23. Jaw1 is an ER-resident molecule (19), while CD23 traffics through the secretory pathway and is expressed at the cell surface. Biochemical and immunocytochemical characterization of Jaw1($NP_{147-155}$) confirmed that it is localized to the ER. Infection of cells with VV-CD23($NP_{147-155}$) resulted in the production of a protein of the expected mobility in

sodium dodecyl sulfate polyacrylamide gel electrophoresis and the surface expression of a protein reactive with a panel of three anti-CD23 monoclonal antibodies (data not shown).

$NP_{147-155}$ was liberated from each protein expressed in T2 cells, demonstrating that the COOH-end rule extends to membrane-bound proteins. The ER localization of Jaw1 suggests that peptides are liberated in the early secretory pathway. This conclusion is supported by the biochemical recovery of the $NP_{147-155}$ peptide from T2 cells co-expressing Jaw1($NP_{147-155}$) and a form of K^d retained in the early secretory pathway (H. L. Snyder et al., submitted for publication).

We next quantitated the liberation of $NP_{147-155}$ from the COOH termini by determining the ability of the various rVVs to enhance K^d expression on the surface of T2 cells. Liberation from secreted NP or CD23 was insufficient to detect enhanced K^d expression. The liberation of $NP_{147-155}$ from Jaw, by contrast, was very efficient—more efficient, in fact, than its liberation from the ER-targeting sequence alone. It will be of interest to determine whether Jaw is in general a better delivery vehicle for antigenic peptides than signal sequences alone.

THE COOH-END RULE

These findings demonstrate that peptides are liberated from the COOH end of both soluble and membrane-bound proteins. Including our previous study (17), all determinants tested in five polypeptide contexts were liberated (nine permutations). Based on these findings, we propose the COOH-end rule: The secretory compartment has a broad capacity to liberate COOH-terminal peptides for association with class I molecules.

There are a number of non–mutually exclusive mechanisms that may contribute to the C-end rule. First, the mere location of a peptide at the COOH terminus of a protein will obviously simplify its liberation by eliminating half of the task of proteolysis. Assuming that proteolytic liberation of NH_2- and COOH-terminal residues is a random process, this would multiply the chance of producing the correct peptide by a factor equivalent to the chance of liberating a single end. If the proteolytic events were rare, this could have a very large effect on peptide liberation. In this case, however, we would expect that the NH_2-terminal peptide of the ER-targeted tandem peptides (17) should be produced at similar efficiency as COOH terminus. Since this was not observed, this argues that other factors play an important role in the C-end rule.

Second, the ER may be an aminopeptidase-rich, carboxypeptidase-poor compartment. This would be consistent with our previous observation that the TAP-dependent presentation of a cytosolic peptide with a carboxy-terminal extension requires the expression of an exotic secretory carboxypeptidase (20). We previously proposed that aminopeptidase activity alone could account for the liberation of the COOH-terminal tandem peptide and the concomitant destruction of the NH_2-terminal peptide (17). While the present findings do not dispel the possible contribution of aminopeptidases to ER processing, it seems highly unlikely that aminopeptidases acting alone can liberate the peptides from the COOH terminus of the 498-residue-long SNP. More conclusively, the liberation of COOH-terminal peptides from membrane-bound proteins demonstrates the presence of endopeptidase activity. Thus, we have to modify this model to include endopeptidases working alone or in concert with aminopeptidases. The presence of endopeptidases would also account for the TAP-independent presentation of peptides from internal locations in polypeptides (9), with the sporadic nature of such events due to the lack of carboxypeptidase activity to further process COOH termini.

Third, the side chain of the COOH-terminal residue of class I binding peptides makes crucial interactions with class I molecules (serving as a "dominant anchor" residue due to its interaction with the pocket), while there is little selection at the NH_2 terminus (not a single class I binding motif features a dominant anchor position at the NH_2 terminus) (5). As originally suggested (21), class I molecules themselves may possess proteolytic activity or may contribute to the proteolytic activity of other ER proteins, either by properly positioning the peptide for cleavage or by recruiting the peptidase to the peptide. Proteins with COOH-terminal extensions may be useful in future studies to catch class I flagrante delicto in the production of antigenic peptides.

Regardless of the underlying mechanism, the COOH-end rule indicates that the ER has a high capacity for removing NH_2-terminal residues from antigenic peptides. Since TAP demonstrates the same specificity for COOH-terminal residues as the class I molecules it feeds (6) and efficiently transports peptides with twice as many residues as peptides most commonly recovered from class I molecules, the present findings suggest a model in which many TAP-transported peptides are trimmed at the NH_2 termini while tenuously associated with class I molecules.

Finally, the COOH-end rule may have evolutionary implications for the composition of proteins in organisms that express class I molecules (essentially all vertebrates). Two factors could mitigate against the presence of COOH-terminal class I binding peptides. First, the interaction of COOH-terminal peptides with class I mol-

ecules may interfere with the function of the protein. Second, the peptides could compete with other class I binding peptides, reducing the efficiency of the class I processing system. These factors may result in the selection of proteins that lack class I binding peptides at their COOH termini or, if such peptides are present, fold rapidly in a manner that prevents access of the peptide to class I molecules. As the number of ER-targeted protein sequences increases from gene sequencing projects, it should be possible to test this idea by comparing the COOH termini between organisms with and without class I molecules.

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Cells Treated with TAP-2 Antisense Oligonucleotides Are Potent Antigen-Presenting Cells In Vitro and In Vivo

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Treatment of RMA and EL4 cells or freshly isolated splenocytes with antisense (AS) oligonucleotides directed against the *TAP-2* gene recreates the phenotype seen in cells that are genetically deficient in TAP function. Cells incubated with AS oligonucleotides exhibit reduced MHC class I expression on the cell surface, which can be increased by incubating the oligonucleotide-treated cells at 28°C or by adding MHC haplotype-matched peptides to the culture medium. RMA cells or splenocytes treated with AS oligonucleotides and incubated with peptide were highly effective in generating primary CTL responses in vitro. The bulk of the AS oligonucleotide-responsive and CTL-inducing cells resided in the adherent fraction of splenocytes. Moreover, TAP-2 AS oligonucleotide-treated adherent splenocytes pulsed with OVA peptide elicited potent OVA-specific CTL responses in vivo and provided effective protection from challenge with tumor cells expressing the corresponding Ag. AS oligonucleotide technology provides a simple approach to develop broadly applicable methods for generating potent APC to study TAP function in normal cells and to identify other gene products involved in MHC class I presentation. *The Journal of Immunology,* **1996, 156: 1772–1780.**

Cytotoxic $CD8^+$ T lymphocytes (CTL) recognize peptides derived from endogenously processed viral, bacterial, or cellular proteins, in association with MHC class I molecules (1). CTL epitopes, consisting of 8 to 10 amino acid (aa)[2] long peptides, are generated from endogenously synthesized proteins in the cytosol, enter the endoplasmic reticulum (ER) where they associate with newly synthesized MHC class I molecules, followed by translocation to the cell surface for presentation to $CD8^+$ T cells (2–4).

Genetic analysis has played an important role in elucidating the pathway of MHC class I-restricted Ag processing and presentation. Studies of Ag presentation of defective human and murine cell lines have demonstrated the requirement of TAP-1 and TAP-2 proteins to transport peptides into the ER, where their association with MHC class I molecules is a prerequisite for class I assembly (5). Mutagenesis of RMA cells, a cell line derived from the Rauscher virus-induced lymphoma of C57BL/6 ($H\text{-}2^b$) origin, and selection for loss of MHC class I expression, led to the isolation of a mutant cell line called RMA-S, which expressed reduced amounts of MHC class I molecules on the cell surface and was unable to present MHC class I-restricted Ags to $CD8^+$ T cells (6–10). The rate of MHC class I synthesis in the mutant RMA-S cells was normal, but newly synthesized MHC molecules were retained in the ER and were not transported to the cell surface (11, 12). The defect in RMA-S cells was pinpointed to a mutation in the *TAP-2* gene whose product forms a heterodimeric complex with the *TAP-1* gene product that functions as a pump to translocate peptides across the ER membrane (3, 5). Binding of peptide to nascent MHC molecules is therefore a prerequisite for the transport of MHC class I molecules to the cell surface and presentation of peptide to CTL.

MHC class I expression on the surface of the TAP-2-deficient RMA-S cells can be induced by incubating the cells at reduced temperatures (12), or by adding $H\text{-}2^b$-restricted peptides to the cell culture, which associate with and stabilize the "empty" MHC class I molecules (8, 10, 13). Consequently, incubation of RMA-S cells with a specific MHC class I-binding peptide creates a high density of a particular MHC-peptide complex on the cell surface (14). De Bruijn et al. have demonstrated that RMA-S cells loaded with a specific peptide are capable of priming CTL responses in vitro, underscoring the importance of epitope density on the cell surface in stimulating naive T cells (15).

Generation of specific mutations and studying their consequences in mammalian cells is usually limited to genes that do not affect long-term viability of the cells, and to established cell lines that can be propagated indefinitely in vitro. Inhibition of gene expression using antisense (AS) oligonucleotides is an alternative and potentially simpler method to inactivate gene function which, due to its immediate and transient nature, is applicable to immortalized as well as primary cells. In this study, we have shown that treatment of tissue-culture established cells such as RMA or EL4 cells, or primary cells such as mouse splenocytes, with AS oligonucleotides directed against the murine *TAP-2* gene, recreates the characteristic phenotype of TAP-2-deficient RMA-S cells. Specifically, cells treated with an AS oligonucleotide targeted to TAP-2 exhibit reduced MHC class I expression at 37°C. MHC expression can, however, be restored by incubating the cells at 28°C, or by addition of the appropriate peptide. Moreover, RMA cells or splenocytes treated with antisense oligonucleotide followed by addition of peptide induce potent, primary CTL responses in vitro and in vivo.

This study offers a novel approach to identify and dissect the function of other gene products involved in the MHC class I presentation pathway, and provides a simple and broadly applicable method for generating primary CTL responses in vitro and in vivo.

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[2] Abbreviations used in this paper: aa, amino acid; ER, endoplasmic reticulum; AS, antisense; DC, dendritic cells; Mø, macrophage; TAP, transporter associated with antigen presentation; HSP, heat shock proteins. IMDM, Iscove's modified Dulbecco's medium; R:S, responder to stimulator.

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Materials and Methods

Mice

C57BL/6 mice (H-2^b), 5 to 7 wk old, were obtained from The Jackson Laboratory (Bar Harbor, ME). In conducting the research described in this paper, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The animal facilities at Duke University Medical Center (Durham, NC) are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Cell lines

RMA and RMA-S cells are derived from the Rauscher leukemia virus-induced T cell lymphoma RBL-5 of C57BL/6 (H-2^b) origin (6). Other cell lines used were EL4 (C57BL/6, H-2^b, and thymoma) and E.G7-OVA (EL4 cells transfected with the cDNA of chicken OVA (16). All cells were maintained in DMEM supplemented with 10% FCS, 25 mM HEPES, 2 mM L-glutamine, and 1 mM sodium pyruvate. E.G7-OVA cells were maintained in medium supplemented with 400 μg/ml G418 (Life Technologies, Grand Island, NY).

Peptides

The following synthetic peptides encoding CTL epitopes in chicken OVA, and influenza nucleoprotein were used: OVA, aa 257–264 SIINFEKL (H-$2K^b$); NP, aa 50–57 SDYEGRLI (H-$2K^k$); NP, aa 147–155 TYQRTRALV (H-$2K^d$); and NP, aa 366–374 ASNENMETM (H-$2D^b$) (17). Peptides were purchased with unblocked (free) amino and carboxyl ends from Research Genetics (Birmingham, AL). Peptides were dissolved in serum-free IMDM and stored at −20°C.

Oligonucleotides

AS oligonucleotides (AS-1 to AS-4), complementary to four different regions of the TAP-2 mRNA were synthesized as 25 nucleotide long phosphorothioate derivatives (Oligos Etc., Wilsonville, OR). The control oligonucleotide (CON-1) was synthesized by reversing the sequence order of oligonucleotide AS-1 to maintain the original base composition. The sequence of the oligonucleotides and their position on the TAP-2 mRNA is shown below (18). Numbering starts at the initiation codon:

AS-1 [25-1]: 5'-AGGGC CTCAG GTAGG ACAGC GCCAT-3'
AS-2 [815-790]: 5'-GCAGC AGGAT ATTGG CATTG AAAGG-3'
AS-3 [1088-1063]: 5'-GTCTA CATCG CTCCA GGGCC TCCTT-3'
AS-4 [1427-1402]: 5'-ACGAA AAGGA GACGT CTTGG AATTC-3'
CON-1: 5'-TACCG CGACA GGATG GACTC CGGGA-3'

Oligonucleotides were dissolved in serum-free medium to make a 100 μM stock, sterilized by filtration through a 0.2-μm cellulose acetate filter, and stored at −20°C.

Antibodies

The mAbs used in this study were as follows: purified anti-mouse H-$2D^b$ (clone 28-8.6) and FITC conjugated anti-mouse H-$2K^b$ (clone AF6-88.5), purchased from PharMingen (San Diego, CA). FITC-conjugated $F(ab')_2$ fragment of donkey anti-mouse IgG (H+L) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Treatment of cells with oligonucleotides

Tumor cells (in log phase) or splenocytes were washed twice in Opti-MEM medium (Life Technologies). Cells were resuspended in Opti-MEM medium at 5 to 10 × 10^6 cells/ml and added to 24-well or 6-well plates. The cationic lipid, Lipofectin (Life Technologies), was used to deliver oligonucleotides into cells as described by Chiang et al. (19). Oligonucleotide and lipofectin were added to Opti-MEM medium at the desired concentration and mixed in a 12- × 75-mm polystyrene tube at room temperature for 20 min. The complex was added to the cells to achieve a final concentration of 400 nM oligonucleotide and 15 μg/ml Lipofectin and incubated at 37°C for 6 to 8 h. The cells were washed, incubated at 28°C or 37°C for 24 to 48 h, and analyzed for MHC class I expression by flow cytometry or used as stimulators for CTL induction.

Acid treatment of cells

RMA cells or splenocytes (2 × 10^7 cells) were irradiated, washed, and gently resuspended in 5 ml of medium containing RPMI 1640, supplemented with 25 mM HEPES and 5% FCS, and adjusted to pH 3 with concentrated HCl (20). Cells were centrifuged immediately and resuspended in IMDM medium supplemented with 10% FCS and 10 μM peptide.

Induction of OVA-specific CTL in vitro

Splenocytes obtained from naive C57BL/6 female retired breeders were treated with ammonium chloride Tris buffer (pH 7.2) for 3 min at 37°C to deplete RBC. The cells were resuspended in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 5 × 10^{-5} M β-mercaptoethanol, and 1 mM sodium pyruvate and enriched for adherent cells by two rounds of adherence at 37°C for 90 min each. Unfractionated splenocytes, adherent cells, and nonadherent cells were treated with oligonucleotide-cationic lipid complex to generate stimulator cells for induction of CTL responses. B cells were separated from the nonadherent population (B and T cells) by panning on anti-Ig coated plates. The separated cell population, which was composed of >80% T lymphocytes by FACS analysis, was used as responder T cells.

Tumor cell lines and splenocytes were treated with oligonucleotide and lipofectin as described above, washed, and incubated for 20 to 24 h at 28°C. Cells were washed, resuspended in IMDM containing 10% FCS and irradiated 7500 rad for RMA or RMA-S cells and 3000 rad for splenocytes. Cells were washed once and precultured for 4 h at 28°C in IMDM with 10% FCS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, 5 × 10^{-5} M β-mercaptoethanol, and 10 μM OVA peptide (or control peptide) prior to use as stimulators for CTL induction.

Naive T cells isolated from C57BL/6 spleens were resuspended in complete IMDM medium at 5 × 10^6 cells/ml and used as responders for primary OVA-specific CTL induction in vitro. A fixed number of T cells, 5 × 10^5 cells/100 μl, were cultured with stimulators (100 μl) at various responder to stimulator (R:S) ratios in 96-well, U-bottom tissue culture plates for 5 days at 37°C. Effectors were harvested after 5 days of culture on a Histopaque 1083 gradient (Sigma, St. Louis, MO).

Induction of OVA-specific CTL in vivo

Tumor cell lines and adherent fraction of splenocytes were washed and treated with oligonucleotide and lipofectin as described above. Cells were washed and resuspended in IMDM containing 10% FCS and irradiated at 20,000 rad for E.G7-OVA and EL4 cells, 7500 rad for RMA cells, and 3000 rad for splenocytes. Cells were washed once and pulsed with OVA peptide or control NP (H-$2D^b$) peptide for 4 h at 28°C in IMDM with 10% FCS and 1 mM sodium pyruvate. After 4 h cells were washed twice and resuspended in PBS for immunizations. Naive, syngeneic C57BL/6 mice were immunized i.p. with 2 × 10^6 cells/mouse in 500 μl PBS. E.G7-OVA and EL4 cells were used at a concentration of 5 × 10^6 cells/mouse.

Splenocytes were harvested after 10 days and depleted of RBC as described. A total of 1.5 × 10^7 splenocytes were cultured with 1 × 10^6 irradiated E.G7-OVA stimulator cells (20,000 rad) in 5 ml of IMDM with 10% FCS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 5 × 10^{-5} M β-mercaptoethanol/well in a 6-well tissue culture plate. Cells were cultured for 5 days at 37°C and 5% CO_2. Effectors were harvested on day 5 on Histopaque 1083 gradient prior to use in a CTL assay.

Tumor protection in vivo

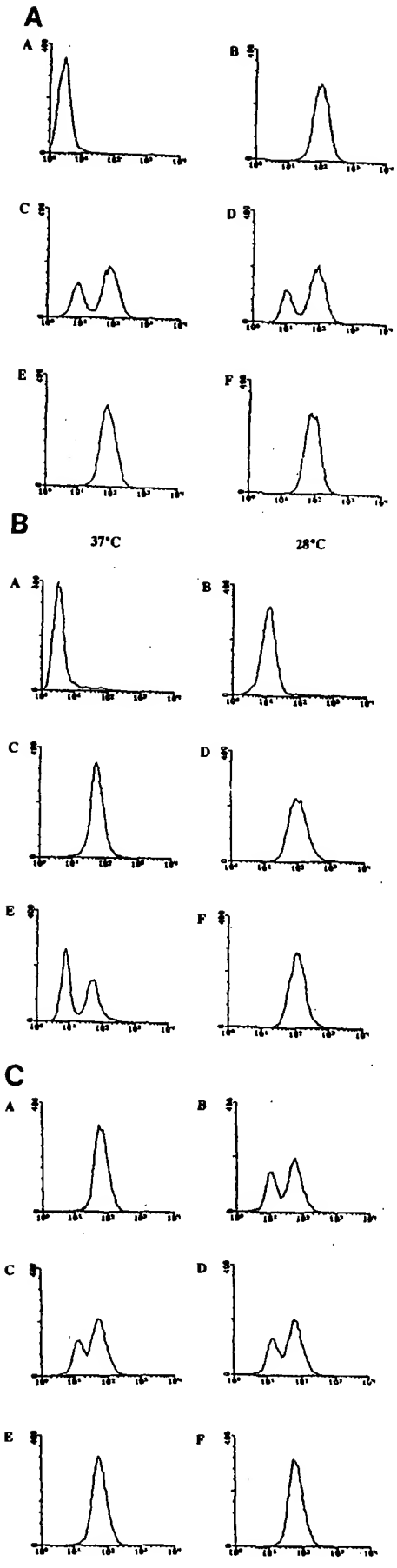
C57BL/6 mice were immunized once with irradiated 2 × 10^6 oligonucleotide treated cells or 5 × 10^6 E.G7-OVA or EL4 cells. Ten days after immunization mice were challenged with 2 × 10^7 live E.G7-OVA cells s.c. in the flank region. Mice were monitored on a regular basis for tumor growth and size. Mice with tumor sizes >3.5 cm were killed. All survivors were killed 40 days after challenge.

Cytotoxicity assay

A total of 5 to 10 × 10^6 target cells were labeled with europium diethylenetriamine pentaacetate for 20 min at 4°C. After several washes, 10^4 europium-labeled targets and serial dilutions of effector cells at E:T ratio of 50:1 to 6.25:1 were incubated in 200 μl of RPMI 1640 with 10% heat-inactivated FCS in 96-well V-bottom plates. The plates were centrifuged at 500 × *g* for 3 min and incubated at 37°C and 5% CO_2 for 4 h. Fifty microliters of the supernatant was harvested and europium release was measured by time-resolved fluorescence (Delta fluorometer, Wallac, Gaithersburg, MD) (21). Spontaneous release was less than 25%. SE of the means of triplicate cultures was less than 5%.

Flow cytometry analysis

Approximately 10^6 cells were incubated in PBS containing 3% BSA with the appropriate concentration of the primary Ab for 30 min at 4°C. The cells were washed and, if required, incubated for 30 min on ice with the secondary Ab, washed, and resuspended in PBS with 3% BSA. Cells stained with isotypic Abs were used as controls. Class I expression was analyzed on a FACScan (Becton Dickinson, Mountain View, CA).



Results

The murine T cell lymphoma, RMA-S, is defective in intracellular processing of MHC class I-restricted peptides due to a point mutation in the *TAP-2* gene (18, 22). The primary aim of this study was to develop a simple method to recreate this phenotype in other cell types while eliminating the need to isolate genetically stable mutant cell lines. To achieve this goal, we treated cells with AS oligonucleotides directed against the TAP-2 mRNA, and then tested whether the observed phenotype was consistent with the predicted phenotype.

The MULFOLD computer program (23) was used to screen mouse TAP-2 mRNA for sequences that are less likely to be sequestered in extensive secondary structure. Four 25 nucleotide long AS oligonucleotides (AS-1 to AS-4) with GC content of 50% to 60% were synthesized as phosphorothioate derivatives to enhance their intracellular stability, as described in *Materials and Methods*. In addition, a control oligonucleotide (CON-1) was synthesized, which represents the reverse, rather than the complement, of the AS-1 oligonucleotide. RMA cells were incubated with the oligonucleotides for 6 to 8 h in the presence of Lipofectin, which was shown in previous studies to enhance oligonucleotide uptake and potentiate the inhibition of gene expression by AS oligonucleotides (19).

Cell lines deficient in transporter function, including RMA-S cells, retain the majority of their MHC class I molecules in the ER in an incompletely assembled form, and hence display reduced amounts of MHC class I molecules on the cell surface (6, 7). Accordingly, we screened the ability of the AS oligonucleotides to inhibit TAP-2 function by analyzing cell-surface expression of MHC class I molecules. As shown in Figure 1*A*, when RMA cells were treated with two of the four AS oligonucleotides, AS-1 and AS-2, MHC class I expression was decreased in approximately 30% of the cells. No effect on MHC class I expression was seen when cells were treated with the two other oligonucleotides, AS-3 or AS-4.

To confirm that the observed reduction in MHC class I expression was indeed due to inhibition of TAP-2 function, we examined

FIGURE 1. Cell surface expression of MHC class I molecules in TAP-2 AS oligonucleotide-treated RMA cells. *A*, MHC class I expression is decreased in cells treated with AS oligonucleotides. RMA cells were incubated in the presence of four AS oligonucleotides directed against different regions of murine TAP-2 mRNA as described in *Materials and Methods*, and analyzed for MHC class I expression. *Panel A*, isotypic control: *Panel B*, untreated RMA cells; RMA cells treated with AS oligonucleotides: AS-1 (*panel C*); AS-2 (*panel D*); AS-3 (*panel E*); and AS-4 (*panel F*). Cells shown in *panels B, C, D, E,* and *F* were stained with FITC-conjugated anti-mouse H-2K^b (clone AF6-88.5). *B*, Incubation at 28°C restores MHC class I expression in RMA cells treated with TAP-2 AS oligonucleotides. RMA cells were treated with TAP-2 AS oligonucleotides, incubated at either 37°C or 28°C, and analyzed for MHC class I expression using FITC-conjugated anti-mouse H-2K^b (clone AF6-88.5). *Panels A* and *B*, RMA-S cells; *panels C* and *D*, RMA cells; *panels E* and *F*, RMA cells treated with AS-1 oligonucleotide; *panels A, C,* and *E*, cells incubated at 37°C; *panels B, D,* and *F*, cells incubated at 28°C. *C*, Incubation with MHC-restricted peptides restores class I expression in RMA cells treated with TAP-2 AS oligonucleotides. RMA cells were treated with the control oligonucleotide CON-1 (*panel A*), or the AS oligonucleotide AS-1 (*panel B*). RMA cells treated with the AS oligonucleotide AS-1 were further incubated with the following peptides described in *Materials and Methods*: NP (H-2K^d), (*panel C*); NP (H-2K^k), (*panel D*); NP (H-2D^b), (*panel E*); OVA (H-2K^b) (*panel F*). Cells shown in panels *A, B, C, D,* and *F* were stained with FITC-conjugated anti-mouse H-2K^b (clone AF6-88.5). Cells in panel *E* were stained with anti-mouse H-2D^b (clone 28-8.6).

the effect of reducing the temperature of incubation and addition of appropriate peptides. As shown in Figure 1*B*, when the TAP-2-deficient RMA-S cells were grown at 37°C, cell-surface expression of MHC class I molecules was very low to undetectable (*panel A*). As previously shown (12), when the temperature of incubation was reduced to 28°C, MHC molecules were re-expressed on the cell surface (*panel B*). Note that even under permissive conditions, expression of class I MHC on RMA-S cells is less as compared parental RMA cells. In contrast to RMA-S cells, expression of MHC class I levels on the surface of the parental RMA cells was not affected by the temperature of incubation (*panels C* and *D*). However, when RMA cells were treated with the oligonucleotide AS-1, MHC expression was reduced in a subpopulation of cells (*panel E*). Note that in this experiment 50 to 55% of the cells were responsive to treatment with the AS oligonucleotide, illustrating the experimental variation of this procedure. As shown in Figure 1*B*, *panel F*, when the AS oligonucleotide-treated RMA cells were incubated at reduced temperatures, MHC class I expression was restored to normal levels.

In the experiment shown in Figure 1*C*, the oligonucleotide-treated cells were incubated with MHC haplotype-matched or -mismatched peptides to determine if addition of exogenous peptide restores MHC class I expression (8, 10). Treatment of RMA cells at 37°C with the AS-1 oligonucleotide, but not with the control oligonucleotide, CON-1, causes reduction of MHC class I expression on approximately 40% of the cells (*panels A* and *B*). As shown in *panels C* and *D*, incubation of the AS-1-treated RMA cells (H-2^b) with H-2^d- or with H-2^k-restricted peptides had no effect on MHC class I expression. In contrast, MHC class I expression was restored to normal levels when the oligonucleotide-treated cells were incubated with either one of two H-2^b-restricted peptides (*panels E* and *F*). We therefore conclude that the observed reduction in MHC class I expression following treatment of RMA cells with AS oligonucleotides directed against TAP-2 was caused by inhibition of TAP-2 function.

MHC class I expression was significantly reduced, but not eliminated, following incubation with the AS oligonucleotides. Based on flow cytometry data shown in Figure 1 we estimate that MHC expression on RMA cells incubated with the TAP-2 AS oligonucleotide is less than 10% as compared with MHC expression on cells treated with the control oligonucleotide. Thus, when MHC expression is restored following incubation with the appropriate peptide, the majority of MHC class I molecules on the surface of the cells are associated with the peptide.

We further examined whether other cell types were susceptible to inhibition of TAP-2 function using AS oligonucleotides. Figure 2 shows the consequences of treating EL4 cells, an established tumor cell line of C57BL/6 origin, with oligonucleotides. Incubation of EL4 cells with the TAP-2 AS oligonucleotide AS-1, but not with the control oligonucleotide, CON-1, causes reduction in MHC class I expression in a subpopulation of cells (Fig. 2, *A–C*). Cell-surface expression of MHC molecules was restored by either incubating cells at 28°C (Fig. 2*D*) or by addition of appropriate peptides (Fig. 2, *E* and *F*). Interestingly, only a fraction of the RMA or EL4 cells, approximately 30% to 60%, responded to treatment with AS oligonucleotides showing a loss of MHC expression in an "all or none" fashion. A possible explanation for this observation is that the turnover of MHC molecules at the cell surface is a cell cycle-dependent phenomenon, thereby affecting only a fraction of the cells during the 6- to 8-h incubation period with the oligonucleotides. (Longer incubation times in the presence of oligonucleotides was toxic to the cells.)

To examine whether TAP-2 function can be inhibited in primary cells, splenocytes from C57BL/6 mice were incubated with TAP-2

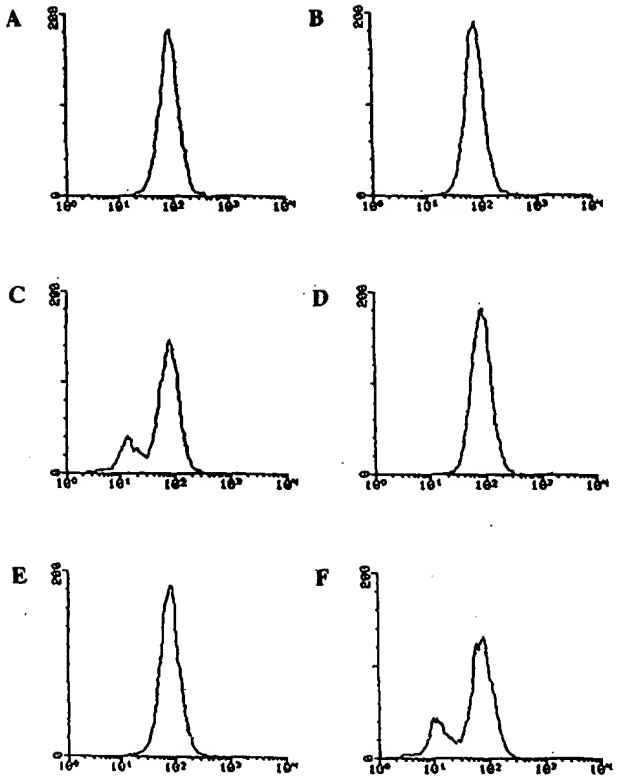


FIGURE 2. MHC class I expression in EL4 cells treated with TAP-2 AS oligonucleotides. Untreated EL4 cells (*A*), EL4 cells treated with the control oligonucleotide CON-1 (*B*), or with the TAP-2 AS oligonucleotide AS-1 (*C*) were analyzed for MHC class I expression. TAP-2 AS oligonucleotide-treated cells were incubated at 28°C (*D*), or incubated with either OVA (H-2K^b) peptide (*E*), or NP (H-2K^k) peptide (*F*). Cells were stained with FITC-conjugated anti-mouse H-2K^b (clone AF6-88.5).

AS oligonucleotides (Fig. 3). In the presence of AS-1 oligonucleotide, about 30% of the splenocytes exhibit reduced MHC class I expression (Fig. 3*C*). However, unlike the pattern seen with RMA or EL4 cells, the extent of MHC class I reduction varied within the affected population, reflecting heterogeneity among the splenocytes in their susceptibility to AS inhibition of TAP-2 function and/or the extent of MHC class I down-regulation as a consequence of TAP-2 inhibition. As seen with RMA cells or with EL4 cells, MHC expression was restored by incubating cells at 28°C (Fig. 3*D*). The observed heterogeneity in splenocytes to treatment with TAP-2 AS oligonucleotides prompted us to test whether the responsive population resided in the adherent fraction, enriched in APC such as monocytes and dendritic cells, or in the nonadherent population enriched in T and B lymphocytes. As shown in Figure 3*E*, over 50% of the adherent population exhibited reduction in MHC class I expression when incubated with the AS oligonucleotide, whereas only a small fraction of nonadherent cells down-regulated MHC expression in the presence of the AS-1 oligonucleotide (Fig. 3*G*). In either case, incubation of the AS oligonucleotide-treated cells at 28°C restored MHC class I expression to normal levels (Fig. 3, *F* and *H*). It is possible that the increased susceptibility of the adherent population to AS inhibition is a reflection of the elevated phagocytic activity of this population. To further confirm the specificity of the AS oligonucleotide we analyzed surface expression of MHC class II, B7-1, B7-2, and intercellular adhesion molecule-1 on the surface of the adherent splenocytes. Treatment of adherent cells with AS oligonucleotide

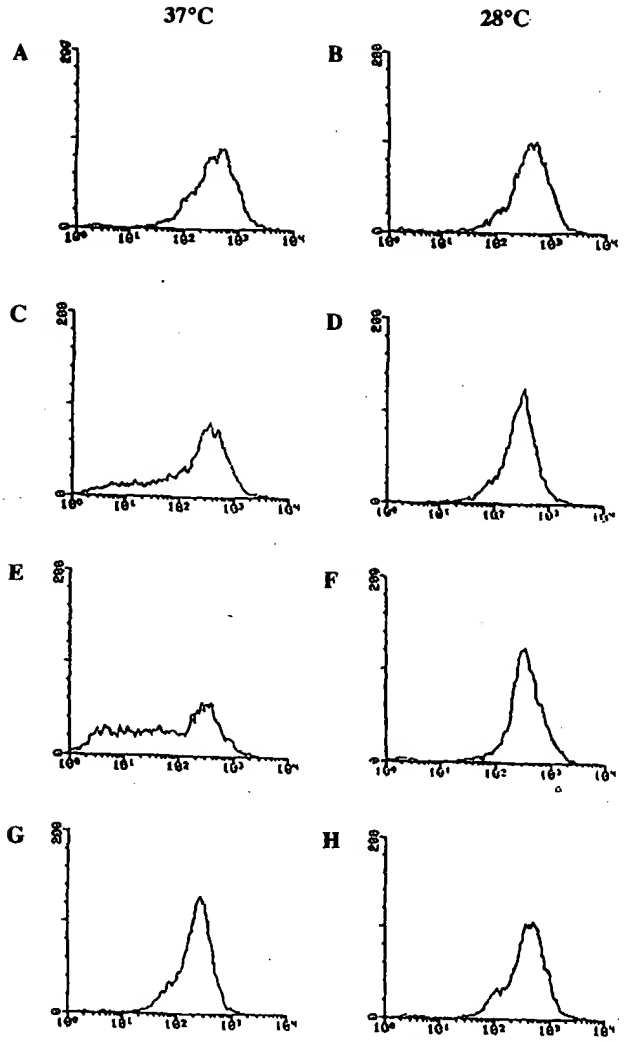


FIGURE 3. MHC class I expression in splenocytes treated with TAP-2 AS oligonucleotides. MHC class I expression in splenocytes isolated from C57BL/6 mice (*A, B*). Unfractionated splenocytes (*C, D*), adherent cells (*E, F*), and nonadherent cells (*G, H*) were treated with the TAP-2 AS oligonucleotide AS-1, and analyzed for MHC class I expression. *A, C, E,* and *G,* Cells were incubated at 37°C; *B, D, F,* and *H,* Cells were incubated at 28°C. Cells were stained with FITC-conjugated anti-mouse H-2K^b (clone AF6-88.5).

had no effect on the expression on any of the above cell-surface molecules (data not shown).

Incubation of TAP-2-deficient cells with appropriate peptide at 37°C results in the loading of the "empty" MHC class I molecules with a specific peptide and the generation of a high density of specific MHC-peptide complexes on the cell surface (14). De Bruijn et al. have shown that RMA-S cells, but not RMA cells, loaded with exogenous peptide are potent APC, capable of inducing primary CTL responses in vitro (15). In the experiment shown in Figure 4, we tested whether RMA cells treated with AS oligonucleotides and incubated in the presence of peptides are able to generate a primary CTL response in vitro. As shown in Figure 4, RMA-S cells, but not RMA cells, pulsed with OVA peptide were capable of inducing CTL from naive T cells against E.G7-OVA cells. However, RMA cells treated with the AS-1 AS oligonucleotide and then incubated with OVA peptide at 28°C stimulated a potent CTL response. No CTL induction was observed when RMA cells were incubated with the control oligonucleotide, CON-1, or when RMA cells were pulsed with MHC-mismatched peptide. No CTL activity was recorded against EL4 cells as targets (data not shown).

In accordance with the above findings, we assessed the ability of AS-treated splenocytes incubated with a specific peptide at inducing primary CTL responses in vitro. As shown in Figure 5, TAP-2 AS oligonucleotide-treated splenocytes incubated with OVA peptide elicited OVA-specific CTL from naive T cells, while treatment with the control oligonucleotide, CON-1, or incubation with MHC-mismatched peptide, did not generate OVA-specific CTL. Consistent with the observation shown in Figure 3, the adherent fraction, enriched in cells responsive to TAP-2 AS inhibition of MHC class I expression, was also more effective in stimulating a primary CTL response compared with the nonadherent fraction or unfractionated splenocytes. Moreover, the adherent, APC-enriched fraction was significantly and reproducibly more effective in generating OVA-specific CTL than RMA cells (data not shown).

The experiments summarized in Figures 4 and 5 show that AS-mediated inhibition of TAP-2 function offers an effective, simple, and broadly applicable method to induce primary CTL responses in a variety of cell types. Langlade-Demoyen et al. have described a simple method to increase the density of specific epitopes on the cell surface, using a mild acid wash to replace the resident peptides bound to the MHC class I molecules with the peptide of choice (24). Such peptide-loaded cells were capable of stimulating a primary CTL response in vitro. Using a similar procedure that did not appear to adversely affect cell viability, we have observed that acid-treated splenocytes, when incubated with OVA peptide, were almost as effective as the AS oligonucleotide-treated cells in generating CTL from naive precursors (Fig. 6*A*). However, when the number of stimulator cells was reduced twofold, the AS oligonucleotide-treated cells were still potent stimulators while the acid-treated cells were barely so (Fig. 6*B*). An additional twofold decrease of AS oligonucleotide-treated stimulators did not significantly decrease their potency to induce CTL (data not shown). The same acid-wash procedure used by Langlade-Demoyen et al. (24) was also less effective than the TAP-2 AS oligonucleotide treatment (data not shown). These results show that on a per cell basis, TAP-2 AS oligonucleotide-treated cells were more effective in stimulating primary CTL responses than acid-treated cells.

We next tested the ability of TAP-2 AS oligonucleotide-treated cells pulsed with OVA peptide to elicit CTL responses in vivo and provide protection against a tumor challenge in C57BL/6 mice. Mice were immunized once with 2×10^6 peptide-pulsed irradiated APCs or with 5×10^6 irradiated E.G7-OVA cells and induction of OVA-specific CTL was determined 7 to 10 days later. In concordance with the primary CTL data shown in Figures 4, 5, and 6, TAP-2 AS-treated adherent splenocytes elicited the highest levels of CTL lysis, which was superior to acid wash treatment (Figure 7). Interestingly, immunization with 5×10^6 E.G7-OVA cells was less effective than immunization with 2×10^6 AS-treated adherent splenocytes. A low level of CTL was generated by injection of adherent splenocytes treated with a control oligonucleotide and pulsed with OVA peptide, presumably reflecting the Ag-presenting properties of macrophages and dendritic cells enriched in this fraction. No CTL were generated in mice immunized with cells pulsed with the control NP (H-2D^b) peptide, EL4 cells, or PBS.

Mice immunized with peptide-pulsed APC or E.G7-OVA cells were challenged with a tumorigenic dose of E.G7-OVA cells 10 days after immunization. Appearance and size of the tumor were determined on a regular basis. Figure 8 shows tumor growth 10 days (Fig. 8*A*) and 35 days (Fig. 8*B*) after tumor implantation. All

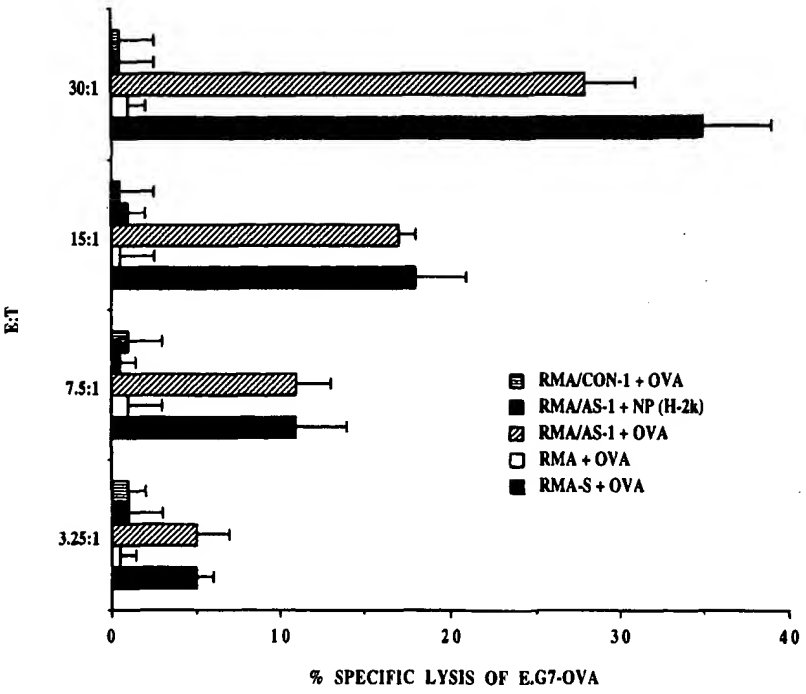


FIGURE 4. Induction of primary, OVA-specific CTL responses using TAP-2 AS oligonucleotide-treated RMA cells. RMA cells treated with AS (AS-1) or with control (CON-1) oligonucleotides and incubated with either OVA (H-2K^b) or NP (H-2K^k) peptides at 37°C as indicated, or RMA-S cells incubated with OVA peptide at 28°C, were used as stimulators. Naive T cells from C57BL/6 (H-2K^b) mice were cultured with stimulators at a R:S ratio of 4:1 for 5 days, and tested for the presence of OVA-specific CTL using E.G7-OVA cells (EL4 cells transfected with the OVA gene) as targets. Lysis of control EL4 cells was less than 2% (data not shown). For additional details, see *Materials and Methods.*

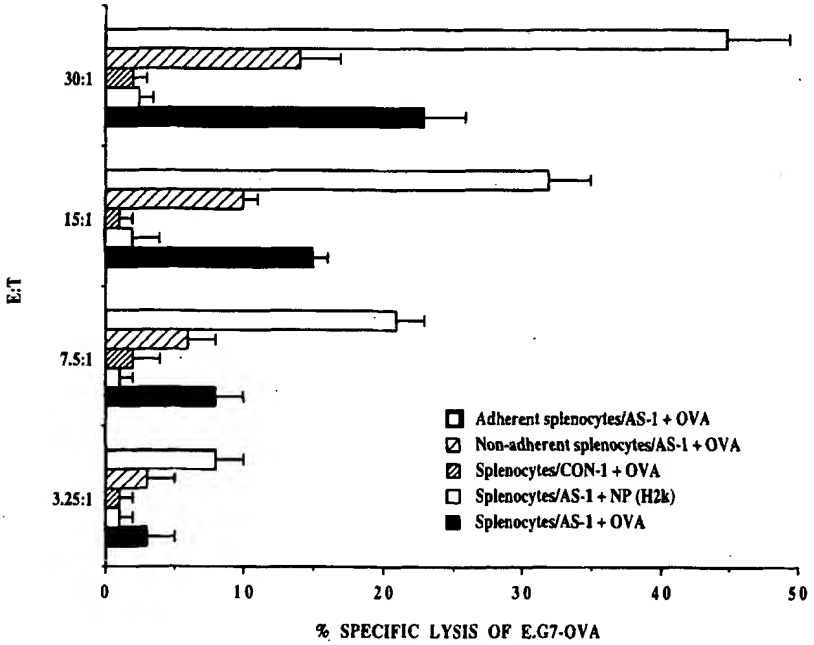


FIGURE 5. Induction of primary, OVA-specific CTL responses using TAP-2 AS oligonucleotide-treated splenocytes. Unfractionated splenocytes obtained from C57BL/6 mice, or splenocytes separated into adherent and nonadherent fractions, were used as stimulators to generate OVA-specific CTL from naive T cells, as described in Figure 4 and *Materials and Methods.*

animals in the control groups developed tumors 3 to 3.5 cm in diameter within 40 days, after which the animals were killed. Immunization with TAP-2 AS oligonucleotide-treated adherent splenocytes provided complete protection from tumor challenge in four out of five mice. In one animal the tumor grew, albeit slowly. Consistent with the CTL data shown in Figure 7, AS-treated RMA cells were less potent than adherent splenocytes. E.G7-OVA tumor cells are highly immunogenic as judged by the fact that one immunization with 10^5 live cells or three immunizations with 5×10^6 irradiated cells elicits a strong CTL response and provides complete protection from a tumor challenge with 2×10^7 E.G7-OVA cells (data not shown). It is therefore noteworthy that a single immunization with 2×10^6 TAP-2 AS-treated adherent splenocytes pulsed with OVA peptide was considerably more effective than one immunization with 5×10^6 irradiated E.G7-OVA cells (Figs. 7 and 8).

Discussion

In this study we demonstrate that treatment of immortalized cell lines or primary cells with AS oligonucleotides targeted against the murine *TAP-2* gene recreates the phenotype of the TAP-2-deficient cell line, RMA-S. First, MHC class I expression was decreased in

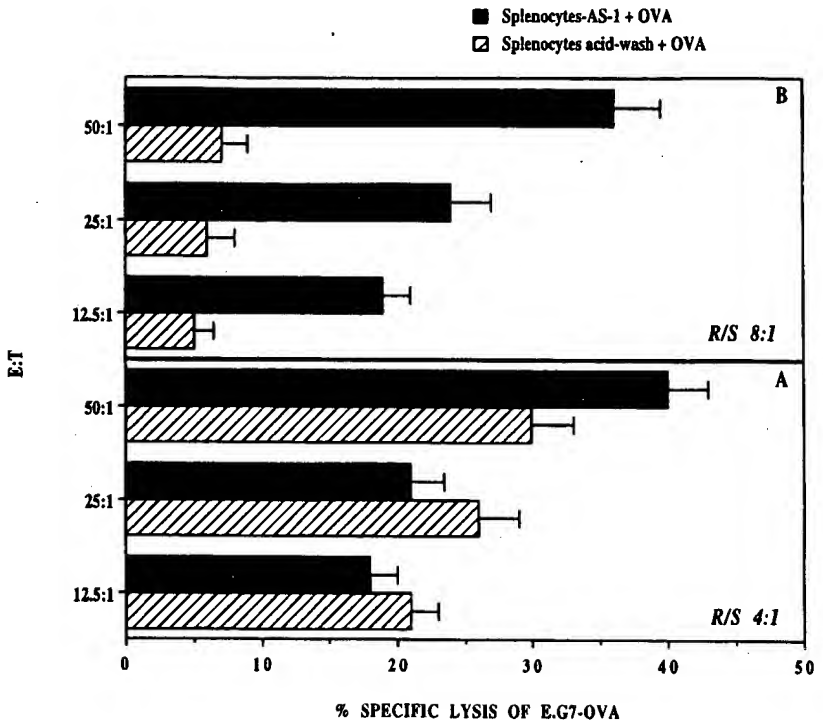


FIGURE 6. Comparison of TAP-2 AS oligonucleotide-treated and acid-washed splenocytes as APC for induction of primary CTL in vitro. Splenocytes were treated with TAP-2 AS oligonucleotide (AS-1), or exposed briefly to pH 3.0 as described in *Materials and Methods*. The oligonucleotide- and acid-treated cells were used as stimulators to generate OVA-specific CTL as described in Figure 4 and *Materials and Methods*. In vitro stimulation was performed at two R:S ratios of 4:1 and 8:1 (5×10^5 responders mixed with 1.25×10^5 or 6.125×10^4 stimulators, respectively).

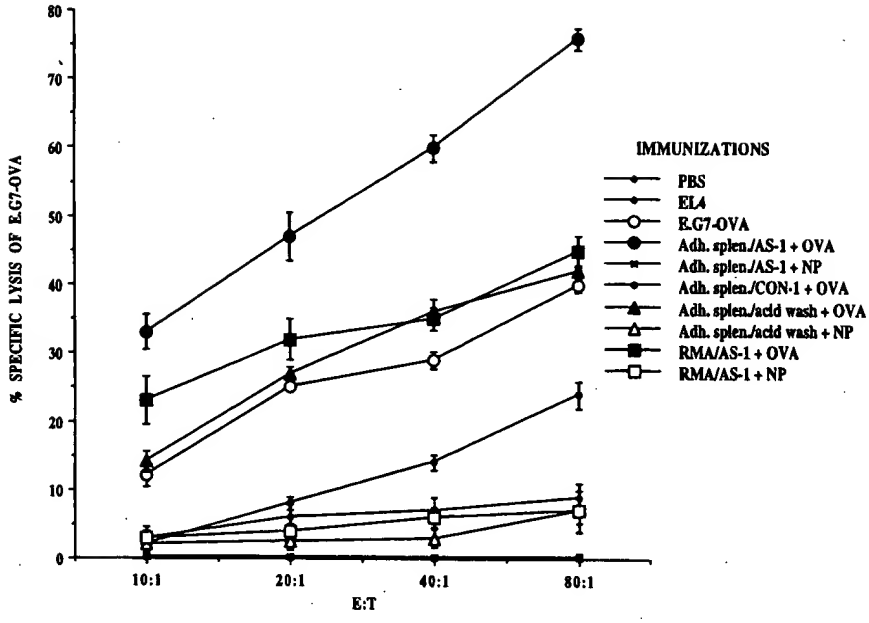


FIGURE 7. Induction of CTL in vivo with a single immunization of TAP-AS oligonucleotide-treated adherent splenocytes. Naive C57BL/6 mice were immunized i.p. with the various cell preparations shown in the figure and as described in *Materials and Methods*. Splenocytes were harvested 10 days later and stimulated with irradiated E.G7-OVA cells (R:S of 15:1) in vitro for 5 days followed by a CTL assay using E.G7-OVA and EL4 cells as targets. Control target, EL4 cells, showed insignificant lysis (data not shown).

cells treated with the AS oligonucleotide (Figs. 1*A*, 2, and 3). Second, incubation of the AS oligonucleotide-treated cells at a reduced temperature restored MHC class I expression to normal levels (Figs. 1*B*, 2, and 3). Third, MHC class I expression was also restored by incubating cells with appropriate haplotype-matched peptides (Figs. 1*C* and 2).

As shown in Figures 4 and 5, RMA cells or splenocytes incubated with AS oligonucleotide and a specific peptide were able to induce a primary CTL response in vitro. Notably, the APC-enriched adherent splenocyte fraction was more effective than RMA-S cells or AS oligonucleotide-treated RMA cells (data not shown). At present, the only cell type other than TAP-2-deficient RMA-S cells that are capable of inducing primary CTL responses in vitro are dendritic cells (25–27). Preliminary results indicate that untreated macrophages and dendritic cells derived from the adherent splenocyte fraction were poor to ineffective stimulators of unprimed $CD8^+$ T cells, respectively. However, when either macrophages or dendritic cells were treated with TAP-2 AS oligonucleotides, they were equally effective in generating primary CTL (data not shown).

Interestingly, although only 40 to 60% of the RMA cells exhibit reduced class I expression, they are comparable with peptide-loaded RMA-S cells in primary CTL responses. One possible explanation is that even under permissive conditions RMA-S cells express 10-fold lower levels of class I MHC as compared with AS-treated RMA cells pulsed with the appropriate peptide (Fig. 1).

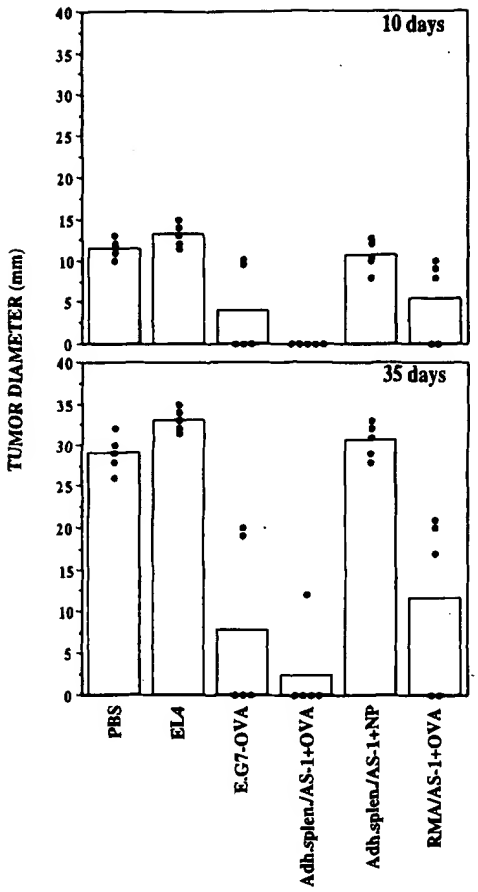


FIGURE 8. Induction of anti-tumor immunity in mice immunized with TAP-2 AS-treated cells. C57BL/6 mice were inoculated i.p. as described in *Materials and Methods* with 2×10^6 AS oligonucleotide-treated cells, or 5×10^6 E.G7-OVA or EL4 cells. Mice were challenged with 2×10^7 live E.G7-OVA cells 10 days after immunization. Results shown are tumor dimensions taken 10 days (*A*) and 35 days (*B*) after challenge. Mice with tumor sizes >3.5 cm in diameter were killed.

Therefore, it is very likely that on a per cell basis AS-treated RMA cells are better APC than peptide-loaded RMA-S cells.

TAP-2 AS oligonucleotide treated cells pulsed with OVA peptide were also highly effective in eliciting CTL and inducing protective anti-tumor immunity in syngeneic mice (Figs. 7 and 8). The exceptional potency of these APC is illustrated by the fact that AS-treated adherent splenocytes were considerably more effective than the highly immunogenic E.G7-OVA tumor cells in generating CTL in vivo (Fig. 7), as well as inducing anti-tumor immunity against a tumor challenge (Fig. 8).

The potency of TAP-2 AS oligonucleotide-treated cells to present Ag to naive cells appears to be a reflection of the high density of specific MHC-peptide complexes on the cell surface. Recently, three reports have described different approaches to increase the density of specific CTL epitopes on the cell surface. Huang et al. used recombinant techniques to generate glycophosphatidylinositol-anchored MHC class I molecules complexed with specific peptides that were incorporated into the outer membrane of cells via the lipid moiety of glycophosphatidylinositol anchor (28). Although the cells were effectively sensitized to lysis by specific CTL, their ability to induce a primary CTL response was not tested. In a second study, Mottez et al. constructed and expressed MHC-peptide fusion proteins on the surface of cells (29). Cells expressing the chimeric MHC-fusion proteins were recognized by specific CTL and were able to induce a primary CTL response in vitro, but only when the cells were cotransfected with a *B7-1* gene construct. Langlade-Demoyen et al. have described a simple method to increase the density of specific epitopes on the cell surface by using a mild acid wash to replace the resident peptides bound to the MHC class I molecules with the peptide of choice, and have shown that such peptide-loaded cells were able to induce primary CTL in vitro (24). However, although the CTL recognized peptide pulsed target cells, their ability to recognize cells expressing the corresponding Ag was not tested. Recognition of endogenously expressed Ag is an important parameter in evaluating the potency of in vitro generated CTL stimulated with peptide-pulsed APC, in view of the observation made by Carbone et al. that immunization with peptide-pulsed cells elicits low affinity CTL incapable of recognizing targets presenting Ag in a physiologic manner (30). Using a procedure similar to one used by Langlade-Demoyen et al. (24), we have shown that the acid-washed cells can be loaded with peptide and generate primary CTL in vitro but less efficiently than TAP-2 AS oligonucleotide-treated cells (Fig. 6). The observed differences between the two protocols can be accounted for by the reported loss of β_2-microglobulin or by other subtle changes that might have occurred in the acid-treated cells (31).

Use of AS oligonucleotides to inhibit TAP-2 function could serve as the basis for developing broadly applicable methods to generate primary CTL in vitro

Induction of CTL from naive precursors has potentially important applications in immunobiology and clinical immunotherapy. Examples are the generation of CTL lines against unknown tumor Ags, which will allow the isolation of the corresponding genes, or the generation and ex vivo expansion of virus and tumor-specific CTL for adoptive immunotherapy. Our studies have shown that TAP-2-inhibited cells are potent inducers of immunity in vivo, and therefore, may have a potentially important role in active immunotherapy.

AS technology offers a simple approach to examine the role of the TAP genes *in normal cells, including primary cells*

Studies that measure the interaction of specific peptides with MHC class I molecules expressed on the cell surface are complicated by the presence of resident peptides derived from endogenously expressed proteins (32–34). Schumacher et al. utilized RMA-S cells expressing "empty" MHC class I molecules at low temperatures to measure the association of specific peptides with MHC class I molecule on the cell surface in the absence of endogenous peptides (14). The ability to recreate the defect of RMA-S cells in other cells could provide a more general approach to measure in situ binding affinity of specific peptides to a wide range of MHC class I molecules. Likewise, it would be of interest to investigate the substrate specificity of allelic variants of the *TAP* gene (35, 36), or the reported existence of a TAP-independent pathway for peptide transport, in professional APC (37–40).

Inhibition of TAP-2 function using AS oligonucleotides provides a paradigm to dissect the function of the various components involved in MHC class I pathway of Ag presentation

Using inhibitors of the major peptidase activities in the cell, Rock et al. have shown that the catalytic degradation of the vast majority of cellular proteins, and generation of most peptides presented on

MHC class I molecules, is the function of a multicatalytic proteinase complex called the proteasome (41). Two subunits of the proteasome, LMP2 and LMP7, have been implicated in the proteolytic generation of MHC class I epitopes, although a recent study does not appear to support this conclusion (42). Using AS oligonucleotides directed against the various components of the proteasome will help to clarify the role of LMP2, LMP7, and the role of other components of the proteasome complex in generating CTL epitopes, as well as their role in the degradation of cellular proteins.

Srivastava and colleagues have shown that antigenic peptides derived from endogenously expressed tumor or viral Ags are complexed with specific heat shock proteins (HSP), gp 96, HSP 90, or HSP 70, and that purified HSP-Ag compexes are capable of stimulating Ag-specific $CD8^+$ T cell responses (43). It was proposed that HPS molecules function as chaperones to facilitate the translocation of processed Ag from the proteasomes to the nascent MHC molecules. Use of AS oligonucleotides directed against the various members of the HSP family will help to elucidate their role in MHC class I Ag presentation.

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Characterization of TAP-Independent and Brefeldin A-Resistant Presentation of Sendai Virus Antigen to CD8$^+$ Cytotoxic T Lymphocytes

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H-2K^b-transfected T2 cells, which lack both TAP1/2 and LMP2/7 genes, are able to efficiently process and present Sendai virus Antigen to K^b-restricted Sendai virus-specific CTL. This presentation is not inhibited by Brefeldin A (BFA). Here we extend our analysis of this novel antigen presentation pathway. We show that presentation of Sendai virus antigen was not due to sensitization of T2K^b cells by peptides in the virus preparation or peptides released from virus infected cells. Also, the ability to present Sendai virus in a BFA resistant fashion was specific for cells of the T2 lineage. Re-expression of TAP1/2 genes in T2K^b cells did not alter the capability to present antigen in a BFA resistant fashion, i.e. the presence of a functional TAP transporter complex did not relocate (all) peptides to the classical pathway for antigen processing and presentation. We found that co-infection of T2K^b cells with either Sendai virus plus influenza virus or Sendai virus plus VSV did not relocate presentation of influenza or VSV antigen to the TAP independent BFA resistant antigen presentation pathway. Peptide elution experiments and studies with peptide-specific CTL firmly demonstrated that the antigen presented by T2K^b cells after infection with Sendai virus was the natural Sendai virus epitope NP324-332. The same epitope, when expressed as a minigene in vaccinia virus, could be presented also by T2K^b cells but this presentation could be blocked by BFA. Thus, the TAP independent BFA resistant presentation of antigen seem cell (T2 lineage) and virus (Sendai virus) specific, but not epitope specific. The ability of T2K^b cells to present Sendai virus antigen in a TAP independent BFA resistant fashion was only partially blocked by lysosomal inhibitors such as methylamine, ammonium chloride and chloroquine. These findings demonstrate that TAP1/2-independent and BFA-resistant class I processing is only expressed in certain cell types, in parallel with classical MHC class I processing, and that Sendai virus selectively can enter this pathway. Hypothetical models for the TAP-independent class I processing are discussed.

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INTRODUCTION

CD8$^+$ cytotoxic T lymphocytes (CTL) kill virus-infected cells by recognizing processed viral peptides in association with MHC class I molecules on the surface of infected cells [1, 2].

Abbreviations: SV, Sendai virus; VSV, vesicular stomatitis virus; PR8, influenza virus A/Puerto Rico/8/34; NP, nucleoprotein; SV9, Sendai nucleoprotein peptide NP324-332; OVA, ovalbumin; VSV8, VSV nucleoprotein peptide NP52-57; BFA, Brefeldin A; VV-SNP$_{M325-332}$, recombinant vaccinia virus encoding Sendai NP325-332; DC, dendritic cells; TAP, transporters associated with antigen processing; LMP, low molecular weight polypeptides.

Viral antigens undergo a limited degradation in which the multicatalytic proteinase complex, or proteasome, may be involved (reviewed in [3]). The resulting peptides are delivered to the lumen of the ER by the help of TAP proteins [4–9]. The class I-peptide complexes are then transported to the cell surface for recognition by CTL [1, 2].

The TAP complex consists of two subunits, TAP1 and TAP2, forming a heterodimer in the membrane of the ER and the cis-Golgi [10–13]. The genes of TAP1 and TAP2 are localized in the class II region of the MHC [14–17] (reviewed in [18]). More recently, it has been demonstrated directly that

the TAP proteins are important in transport of cytosolic peptides into the ER in an ATP-dependent and peptide-selective manner [4–9]. Human TAP and rat TAP^a translocate peptides with hydrophobic and basic C termini, whereas mouse TAP and rat TAP^u selectively transport peptides with hydrophobic C termini with a usual size of 8–14 residues [8, 9].

Several reports have challenged the absolute requirement for the TAP proteins in class I-restricted antigen presentation [19–22] (reviewed in [23]). Peptide presentation restricted by class I molecules apparently can be achieved in macrophages after phagocytosis of recombinant bacteria containing an ovalbumin (OVA) fusion protein [19] as well as in TAP-deficient T2 cells after peptide minigene transfection [20] or HIV-1 envelop protein uptake [21]. We have observed previously that the antigen processing mutant T2 cells, which lack both TAP1/2 peptide transporters and LMP2/7 proteasome subunits, and thus are unable to present endogenously synthesized antigens [24–26], are killed readily by Sendai-specific $CD8^+$ CTL after K^b transfection and viral infection [22]. Intriguingly, the presentation of Sendai virus antigen in $T2K^b$ cells is not blocked by BFA, as was also reported for OVA recombinant *Escherichia coli* or *Salmonella typhimurium* bacteria [19], indicating the presence of an alternate pathway for presentation by class I molecules.

To characterize further the TAP-independent and BFA-resistant presentation of Sendai virus antigen, we have analysed the presentation in different cell types, the effect of endosomal/lysosomal inhibitors, and the influence of Sendai virus on presentation of influenza virus and VSV antigens. We have investigated also whether an immunodominant Sendai epitope (NP324-332) is processed in $T2K^b$ cells and whether the same epitope, if expressed as a vaccinia-based minigene, is presented in the BFA-sensitive or -resistant pathways.

MATERIALS AND METHODS

Cell lines and culture conditions. EL-4 ($H\text{-}2^b$) is a thymoma cell line derived from C57BL/6 mice. $P815K^b$, $HYM2K^b$ and $JurkatK^b$ cells are: P815 (mastocytoma of DBA/2, $H\text{-}2^d$), HYM2 (EBV-transformed human B lymphoma) and Jurkat (human T lymphoma) cell lines transfected with $H\text{-}2K^b$ genes, respectively. T1 is a hybrid of the B lymphoblastoid cell line 721.174 and the T cell line CEM®.3. T2 is selected from T1 cells for loss of CEM®.3-derived chromosome 6 and has a large deletion in the MHC class II region including genes for TAP1/2 and LMP2/7 [27, 28]. $T1K^b$, $T2K^b$ and $T2K^bTAP1/2$ ($T2K^b$ cells transfected with rat TAP1/2) [29] are T1, T2 and T2TAP1/2 cells transfected with $H\text{-}2K^b$ genes. $P815K^b$ was a gift from Dr H. Stauss (University College, London). $JurkatK^b$ was kindly provided by Dr L. A. Sherman (The Scripps Research Institute, La Jolla, CA, USA) via Dr H. N. Eisen (MIT, Cambridge, MA, USA). $HYM2K^b$, $T1K^b$, $T2K^b$ and $T2K^bTAP1/2$ transfectants were generated by Drs F. Momburg and G. J. Hämmerling (German Cancer Research Center, Heidelberg, Germany) and characteristics of $T1K^b$, $T2K^b$ and $T2K^bTAP1/2$ have been described [30]. EL-4 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, penicillin and streptomycin. All transfectants were cultivated in RPMI 1640–10% FCS medium and Geneticin (G-418 Sulfate, 0.5 mg/ml) (Gibco, BRL, UK). Expression of transfected MHC class I genes were regularly checked by FACS analysis and virus-specific CTL assay.

Mice, viruses and synthetic peptides. C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) female mice were purchased from B & K Universal AB (Stockholm, Sweden). All mice were used at the age of 6–10 weeks. Non-virulent Sendai virus, lot 40340087, was obtained originally from Flow Laboratories and was generously provided by Drs W. M. Kast and C. J. M. Melief (University Hospital, Leiden, The Netherlands). Influenza virus A/Puerto Rico/8/34 (PR8) was a gift from Dr A. Douglas (National Institute for Medical Research, London, UK). The Sendai virus and the influenza virus were grown in the allantoic cavity of embryonated chicken eggs and used as allantoic fluid for priming and infecting target cells. VSV Indiana serotype was a gift from Dr K. Kristensson (Huddinge University Hospital, Karolinska Institute, Stockholm, Sweden). The VSV was grown in Vero cells and plaqued on BHK-21 cells. The recombinant vaccinia virus encoding NP325-332 ($VV\text{-}SNP_{M325\text{-}332}$) ([31] it had an error in sequence numbering $VV\text{-}SNP_{M321\text{-}328}$) (*M*APGNYPAL, a methionine for initiation and also for substitution of phenylalanine) was generously provided by Dr J. R. Bennink (National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA). The $VV\text{-}SNP_{M325\text{-}332}$ was grown and plaqued in BHK-21 cells. The Sendai nucleoprotein (NP) peptide 324-332 (FAPGNYPAL, K^b-binding, SV9) [32, 33], VSV nucleocapsid (N) peptide 52-57 (RGYVYQGL, K^b-binding, VSV8) [34] and influenza NP peptide 147-155 (TYQRTRALV, K^d-binding, influenza NP147-155) [35] were synthesized by Drs B. R. Srinivasa (Astra Research Center, Bangalore, India) and U. Rudén (SMI, Stockholm) using f-moc synthesis on a peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA, USA) and the manual 'tea-bag' solid phase method. Peptides were purified by reverse phase HPLC. Stock solution of peptides were prepared in PBS and stored at −20°C.

Isolation of antigenic peptides by affinity purification and HPLC fractionation. $T2K^b$ cells (5×10^8) were infected with 40 ml Sendai virus (2560 HAU/ml) for 1.5 h. After washing, cells were incubated for 1.5 h for monitoring infection by CTL assay or 7.5 h for isolation of peptides. After completion of incubation, cells were washed three times with PBS and frozen at −20°C as a pellet. The same amount of uninfected cells were used as control. Affinity purification of MHC class I molecules and HPLC fractionation of this material were done as described [36]. Briefly, infected and non-infected $T2K^b$ cells were lysed by stirring the cells for 60 min in 20 ml PBS containing 0.5% NP40. The lysates were centrifuged at 23,400 *g* for 30 min using a Sorvall RC-5C centrifuge and a SS34 rotor (Sorvall Instruments, Du Pont, Instrument AB Lambada, Stockholm, Sweden). The pH of the supernatants was adjusted to 8.0 by adding 1 M Tris-HCl buffer pH 8.0. The lysates were pre-cleared by running through columns with Pharmacia 4CL-protein A-Sepharose beads loaded with antibodies from normal mouse serum. In a second step, the pre-cleared lysates were run through an immunoaffinity column specific for $H\text{-}2K^b$ (4CL-protein A-Sepharose beads loaded with AF6-88.5.3, K^b $\alpha1/\alpha2$ specific MoAb). Prior to elution of the bound MHC class I molecules, the columns were washed with 10 mM Tris-HCl buffer pH 8.0. Loaded beads were subject to acid extraction using 100 mM glycin pH 3.0. Bed volumes, 2 ml; flow rates, 0.5 ml/min. Trifluoroacetic acid (TFA) was added to the collected material to a final concentration of 0.1%, which then was incubated for 15 min swirling. All work was performed at 4°C. The collected material was separated on HPLC.

Separation by reverse phase HPLC. The separations were performed on a reverse phase Superpac Pep S column (C2/C18, 5 mM particles, 4.0 × 250 mm; Pharmacia LKB, Uppsala, Sweden) using Pharmacia LKB equipment. Elution procedures: solution A, 0.1% TFA in H_2O; solution B, 0.1% TFA in acetonitrile; 0–5 min, 100% A; 5–45 min, linear increase to 60% B; 45-50 min, 60% B; 50-55 min, linear decrease to 0% B. Flow rate 1 ml/min. Elution was monitored by measuring UV-light absorption at 214 nm in a continuous flow detector, and 1 ml fractions were collected. Individual fractions were dried by vacuum centrifugation.

Generation of virus- and peptide-specific CTL. Generation of virus-specific CTL was done as described [25, 37]. Mice were immunized i.v. or i.p. with 20–100 HAU of Sendai virus or PR8 or i.p. with 10^6 PFU of VSV. After 1–2 weeks, immune spleen cells (25×10^6) were restimulated with virus-infected syngeneic spleen cells (2000 rad, 25×10^6) in 50-ml tissue culture flasks (Costar, Cambridge, MA, USA) with 15 ml RPMI 1640 complete medium (supplemented with 10% FCS, L-glutamine, non-essential amino acids, sodium pyruvate and antibiotics) for 5 days at 37°C, 5% CO_2. The optimal conditions for generation of peptide-specific CTL have been described in detail [37, 38]. Mice were immunized s.c. with 100 mg peptide emulsified in incomplete Freund's adjuvant (IFA). After 7–10 days, immune spleen cells were restimulated with irradiated syngeneic spleen cells in the presence of low concentration of peptide (0.05 mM) for 5 days.

CTL assay. A standard ^{51}Cr-release assay was performed. Preparation of target cells were done by infecting cells ($1–2 \times 10^6$) with virus or incubation with peptides (50 μM) for 1.5 h at 37°C. After virus infection, cells were washed and further incubated for 2–4 h. Target cells were labelled with ^{51}Cr (100 mCi, Dupont, Boston, MA, USA) for 1 h, washed, adjusted to the appropriate concentration and mixed with titrated effector CTL in V-bottomed 96-well plates and incubated for 4–6 h at 37°C. Supernatants were collected and radioactivity measured in a gamma-counter. Results are expressed as percentage of specific lysis according to the formula: % Specific lysis = [(experimental release-spontaneous release) / (maximal release-spontaneous release)] cpm × 100. Vacuum-dried HPLC fractions were dissolved in 200 μl of distilled water and aliquots of 10 μl were plated into V-bottomed 96-well plates. ^{51}Cr-labelled target cells (1×10^4) were added to each well for 30 min incubation at 37°C, and followed by addition of effector CTL. All CTL experiments were reproduced at least three times and representative experiments are shown in the Results section.

Inhibitor treatment. Brefeldin A (BFA) was kindly provided by Sandoz AB (Täby, Sweden), dissolved in methanol (10 mg/ml) and stored at −20°C. Methylamine, ammonium chloride and chloroquine were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in RPMI 1640 medium. Treatment of target cells with BFA or lysosomotropic agents were described [39–45]. Cells were pre-treated with BFA (10 mg/ml) or methylamine (5 mM) or ammonium chloride (20 and 5 mM) for 30 min and infected with virus or incubated with peptide for 1.5 h in the presence or absence of inhibitors. After washing, cells were further incubated for 2–4 h with or without inhibitors before labelling with ^{51}Cr and the CTL assay. The inhibitors were present in the CTL assay.

RESULTS

BFA-resistant presentation of Sendai virus antigen in T2K^b cells

We have found previously that Sendai virus antigen can be processed in T2K^b cells for K^b presentation and that this function is resistant to BFA ([22] and Fig. 1). The possibility that T2K^b cells might be sensitized by contaminating peptide in the Sendai virus preparation has been excluded by the separation of the virus preparation into a low molecular weight fraction (Table 1). No target cell sensitization was seen using the low molecular weight (below 10 kDa) fraction. Furthermore, T2K^b cells did not release K^b-binding peptides after Sendai virus infection as no killing of uninfected ^{51}Cr-labelled by-stander T2K^b cells was seen (Table 2).

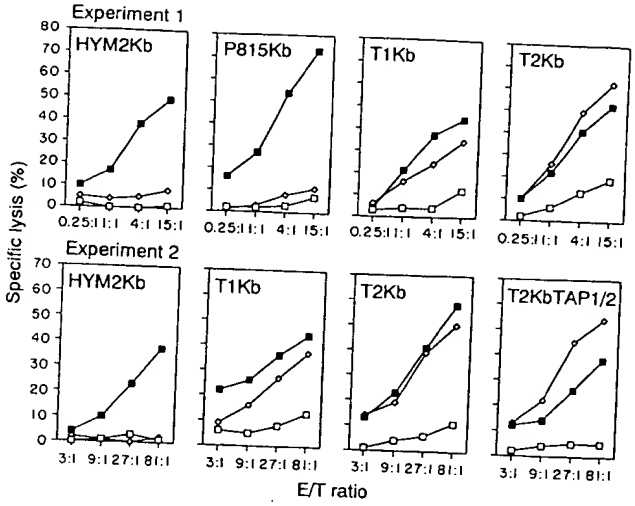

Fig. 1. BFA-sensitive and -resistant presentation of Sendai virus antigen in different cell lines. HYM2K^b, P815K^b, T1K^b, T2K^b and T2K^{b}TAP1/2 cells were left untreated, infected with Sendai virus alone (500 HAU), or with virus in the presence of BFA and assayed for specific CTL lysis. –□–, none; –■–, SV; –◇–, SV + BFA.

Table 1. No peptide contamination in the Sendai virus preparation

Target cells	Treatment[a]	CTL killing[b] 30 : 1	10 : 1	3 : 1
EL-4	untreated	2	1	1
	SV	49	23	12
	SV-filtered	2	1	1
T2K^b	untreated	8	6	4
	SV	49	27	18
	SV-filtered	5	3	2

[a] Target cells were infected with Sendai virus (SV) (250 HAU) or the low mw (10 kDa cut-off) fraction of filtered SV and tested for CTL lysis. To generate the low mw fraction, Sendai virus (corresponding to 250 HAU) was loaded on a 10 kDa cut-off membrane separator and centrifuged at 4300 *g* for 3 h at 4°C.

[b] Per cent specific lysis at the respective E:T ratios indicated.

Table 2. No release of peptide from Sendai virus-infected $T2K^b$ cells

Target cells	CTL killing[a] 27:1	9:1	3:1
^{51}Cr-$T2K^b$	8	2	2
^{51}Cr-$T2K^b$-SV	73	63	49
^{51}Cr-$T2K^b$-SV9	84	82	70
$T2K^b$-SV(cold) + ^{51}Cr-$T2K^b$(hot)	4	2	0

[a] Per cent specific lysis at the respective E:T ratios indicated.

BFA-sensitive and -resistant presentation of Sendai virus antigen in different cell lines

Different cell lines were tested for BFA-sensitive and resistant Sendai virus antigen processing and presentation to Sendai virus specific CTL (Fig. 1). The presentation in K^b-transfected HYM2 (B-LCL) and P815 (mastocytoma) cells was inhibited by BFA, suggesting that only the classical MHC class I processing pathway was operative in these cells. In contrast, presentation of Sendai virus antigen cannot be blocked by BFA in either $T1K^b$, $T2K^b$ or $T2TAP1/2K^b$ cells. Since $T1K^b$ as well as $T2TAP1/2K^b$ cells express TAP1/2 peptide transporters, and can present endogenous antigen via a functional classical MHC class I processing pathway [30], these results demonstrate that the non-classical, BFA-resistant MHC class I processing occurs also in these parental cells and that these two pathways can operate in parallel. Possibly, there is a partial inhibition of Sendai virus antigen processing by BFA in $T1K^b$, which may not be seen in TAP1/2-transfected $T2K^b$ cells (Fig. 1). Nonetheless, the data suggest that different cell types can express different MHC class I processing pathways either selectively or in parallel.

Co-infection of Sendai virus with VSV or influenza virus

In order to assess whether the presence of Sendai virus could induce processing and presentation of VSV or PR8 derived viral antigens in T2 cells (neither of these viruses are presented in $T2K^b$ or $T2K^d$ cells, respectively, [22, 25, 30], we infected $T2K^b$ or $T2K^d$ cells with VSV or PR8 virus alone or co-infected these with titrated doses of Sendai virus. No presentation of VSV or PR8 antigens in $T2K^b$ or $T2K^d$ cells was observed, even if these viruses were co-infected with 1000 HAU of Sendai virus. As expected, control $T1K^b$, $T1K^d$ or P815 cells efficiently presented endogenously synthesized VSV or PR8 antigens (Fig. 2, data not shown). In conclusion, the presence of Sendai virus was not sufficient to re-target presentation of VSV and influenza antigens into the TAP-independent class I processing pathway.

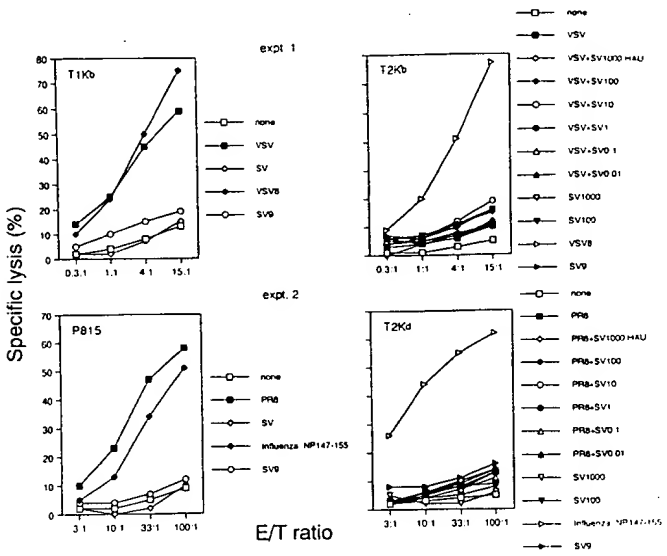

Fig. 2. Co-infection of Sendai virus with VSV or influenza PR8 in $T2K^b$ or $T2K^d$ cells. Expt. 1: $T1K^b$ and $T2K^b$ cells were untreated, infected with VSV (2 PFU), coinfected with VSV (2 PFU) and titrated doses of Sendai virus ranging from 1000 to 0.01 HAU or incubated with the VSV8 peptide, and tested for cytolysis by VSV CTL. Expt. 2: P815 and $T2K^d$ were untreated, infected with PR8 alone (640 HAU), co-infected with PR8 and titrated concentrations of Sendai virus or incubated with the influenza peptide NP147-155, and assayed for anti-PR8 CTL killing.

CTL recognition of HPLC fractions from Sendai virus-infected $T2K^b$ cells

In order to identify the Sendai virus epitope presented by $T2K^b$ cells after infection with Sendai virus, $T2K^b$ cells were infected with Sendai virus and H-$2K^b$ molecules were affinity-purified. After acidic elution, the peptide materials were further separated by reverse phase HPLC and 40 fractions

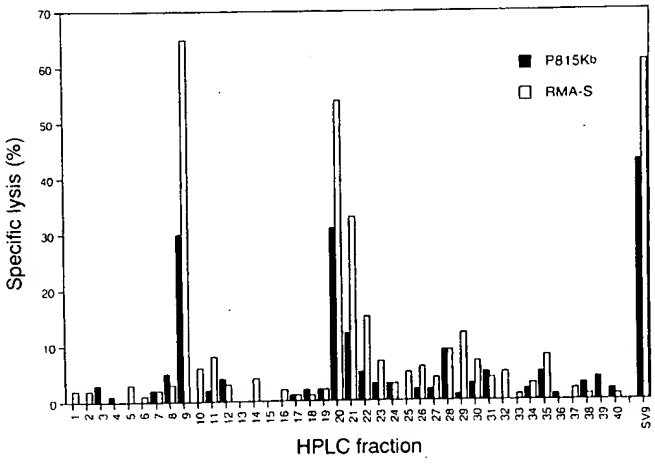

Fig. 3. Recognition of two major HPLC fractions eluted from Sendai virus-infected $T2K^b$ cells by Sendai virus-specific CTL. Target cells were $P815K^b$ and RMA-S. A similar profile was also seen in $T2K^b$ target cells (data not shown). E/T ratio, 50:1.

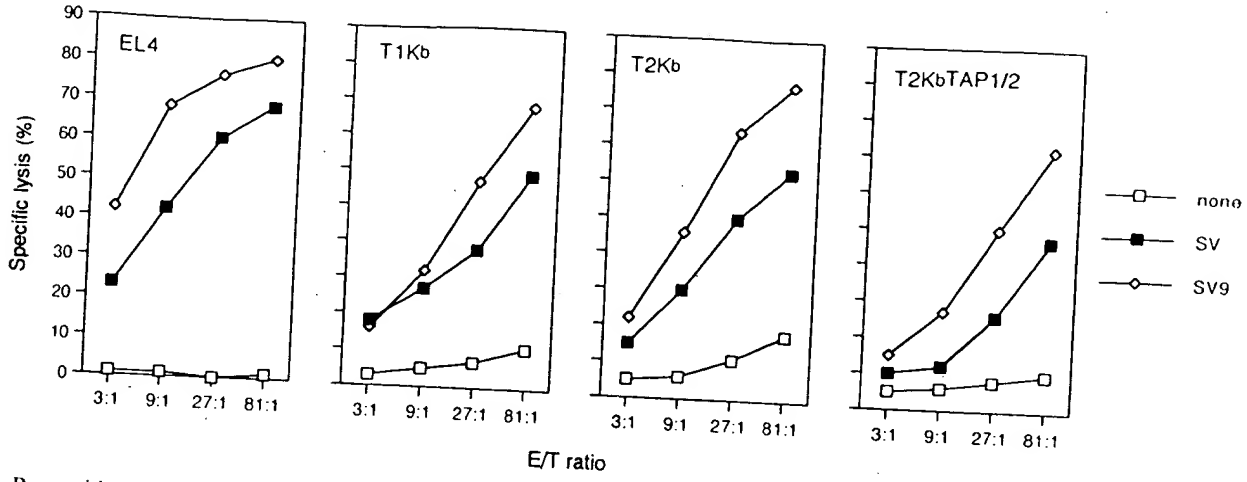

Fig. 4. Recognition of Sendai virus-infected T2K^b cells by Sendai peptide-specific CTL. Peptide-peptide CTL, primed with peptide *in vivo* and restimulated with peptide *in vitro*. Sendai virus-peptide CTL (primed with Sendai virus and restimulated with peptide) were used as control (data not shown).

were collected. Two active fractions (Fig. 3, No.9 and 20) were found strongly to sensitize P815K^b, RMA-S as well as T2K^b (data not shown) target cells for Sendai virus-specific CTL. The synthetic SV9 peptide (NP325-332) eluted was close to fraction 20. This suggested that the Sendai virus antigen presented by H-2K^b in T2 cells, as expected, was of peptide nature and resembled the natural Sendai NP epitope presented by non-mutant H-2K^b expressing cells after Sendai virus infection.

Recognition of Sendai virus-infected T2K^b cells by peptide-specific CTL

Recently, we have defined optimal conditions for generation of CTL responses with short synthetic peptides. Such peptide-induced CTL show a high specificity of target cell recognition and exert a strong killing activity against virus-infected target cells [37, 38]. This allowed us to generate CTL specific for the Sendai virus peptides NP324-332. Such CTL efficiently killed T2K^b as well as control cells infected with Sendai virus (Fig. 4), demonstrating directly that T2K^b cells could process the Sendai virus NP324-332 antigen for presentation to peptide specific CTL.

Presentation of the Sendai minigene epitope in T2K^b cells

EL-4 (H-2^b), JurkatK^b and T2K^b target cells were infected for a short (3 h) or long (12 h) time with recombinant vaccinia virus encoding Sendai NP325-332 (VV-SNP$_{M325-332}$) and tested for their antigen presenting ability to CTL. Figure 5

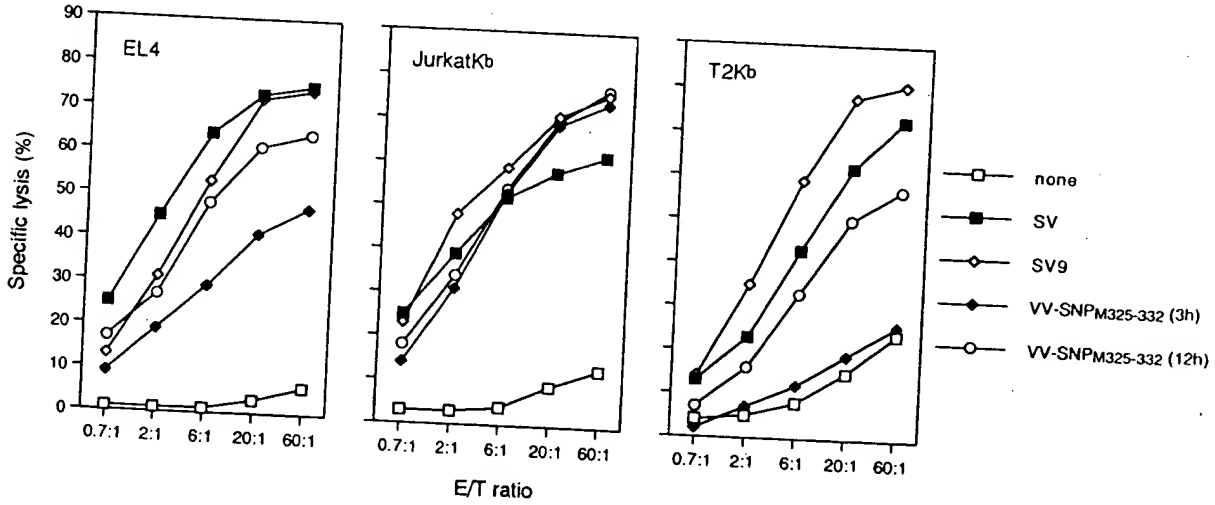

Fig. 5. T2K^b cells presented the minigene-derived peptides after prolongation of infection. EL-4, JurkatK^b and T2K^b cells were untreated, infected with Sendai virus (500 HAU) or VV-SNP$_{M325-332}$ (200 PFU, 3 h or 12 h), or incubated with SV9 and tested for CTL killing. Target cells infected with the vaccinia vector alone were not killed (data not shown).

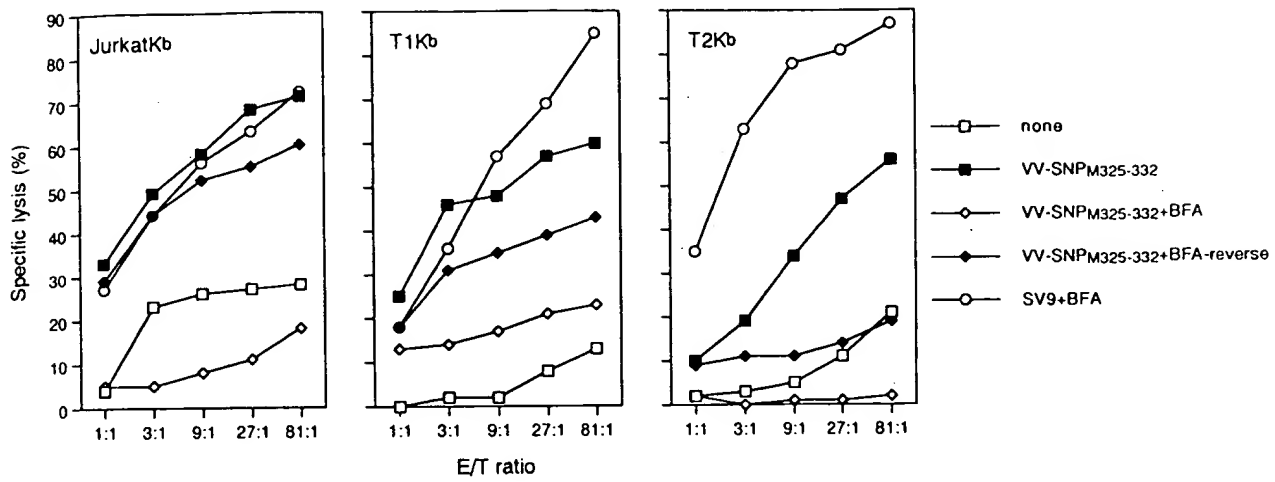

Fig. 6. BFA blocked the presentation of the endogenously minigene-derived peptides in T2K^b cells. JurkatK^b, T1K^b and T2K^b cells were untreated, infected with VV-SNP$_{M325-332}$ (12 h) or incubated with SV9 in the absence or presence of BFA. The BFA blocking effect was reversible. Target cells infected with the vaccinia vector alone were not killed (data not shown).

shows that T2K^b cells after infection with VV-SNP$_{M325-332}$ for 3 h were unable to present the endogenous minigene epitope to Sendai virus-specific CTL. However, after prolongation of infection to 12 h, a significant cytolysis of T2K^b cells was observed. Control EL-4 and JurkatK^b cells, with normal TAP expression, could efficiently present the minigene peptide even after a short time of infection. To exclude that a long time (12 h) of viral infection may lead to a cytopathic effect and thus release a peptide that contributes to sensitize innocent target cells, cell viability was checked. After infection with VV-SNP$_{M325-332}$ for 12 h the cell viability was not affected as determined by trypan blue staining (data not shown). Furthermore, the supernatants from VV-SNP$_{M325-332}$-infected (12 h) T2K^b cells did not sensitize target cells for lysis by Sendai virus CTL (data not shown). Thus, T2K^b cells, after prolonged infection, could present the Sendai minigene epitope independent of TAP expression.

Presentation of the Sendai minigene epitope in T2K^b cells was blocked by BFA

To understand whether the Sendai minigene epitope follows a similar BFA-resistant presentation pathway as Sendai virus in T2K^b cells, we assessed the effect of BFA on the presentation of the minigene epitope (Fig. 6). T2K^b cells as well as the wild-type T1K^b cells presented the Sendai minigene epitope NP325-332. For both cells, this presentation could be blocked by BFA (Fig. 6). This result suggested that the TAP-independent presentation of the Sendai minigene epitope in T2K^b cells could occur, but that it was possible to block this presentation with BFA. Thus, the presentation of the Sendai virus epitope NP325-332 follows two different pathways dependent on where the epitope is expressed.

Effect of lysosomal inhibitors on the presentation of Sendai virus antigen in T2K^b cells

To study whether the endosomal/lysosomal compartments with low pH were involved in processing of Sendai virus antigen, methylamine, ammonium chloride and chloroquine were used. Rock and colleagues have described that methylamine and ammonium chloride inhibits class I- and class II-restricted antigen presentation, and in contrast, that chloroquine markedly inhibited class II but not class I-restricted antigen presentation [41]. Figure 7A and B showed that methylamine and ammonium chloride had a weak inhibitory effect on the presentation of Sendai virus antigen in T2K^b as well as in EL-4 cells. As expected, these drugs strongly inhibited the presentation of influenza virus antigen in EL-4 cells as endosomal fusion steps, which are necessary for influenza virus infection, are affected by these drugs (Fig. 7A and B [44]). The concentrations of the drugs used in the present study showed no toxicity in the course of the assay on effector cells as target cells were killed in the presence of exogenously provided peptides (data not shown).

DISCUSSION

In the study described here we made the following observations. First, the TAP-independent and BFA-resistant presentation of Sendai virus antigen was detected only in the T1K^b, T2K^b and T2K^{b}TAP1/2 cells. Second, the presence of Sendai virus was not sufficient to redirect VSV and influenza virus antigen into the TAP-independent and BFA resistant class I processing pathway. Third, the Sendai virus epitope presented by H-2K^b in T2 cells appear to be identical to the epitope NP325-332 presented by non-mutant cells of the H-2^b haplo-type; CTL recognized Sendai virus-infected T2K^b cells.

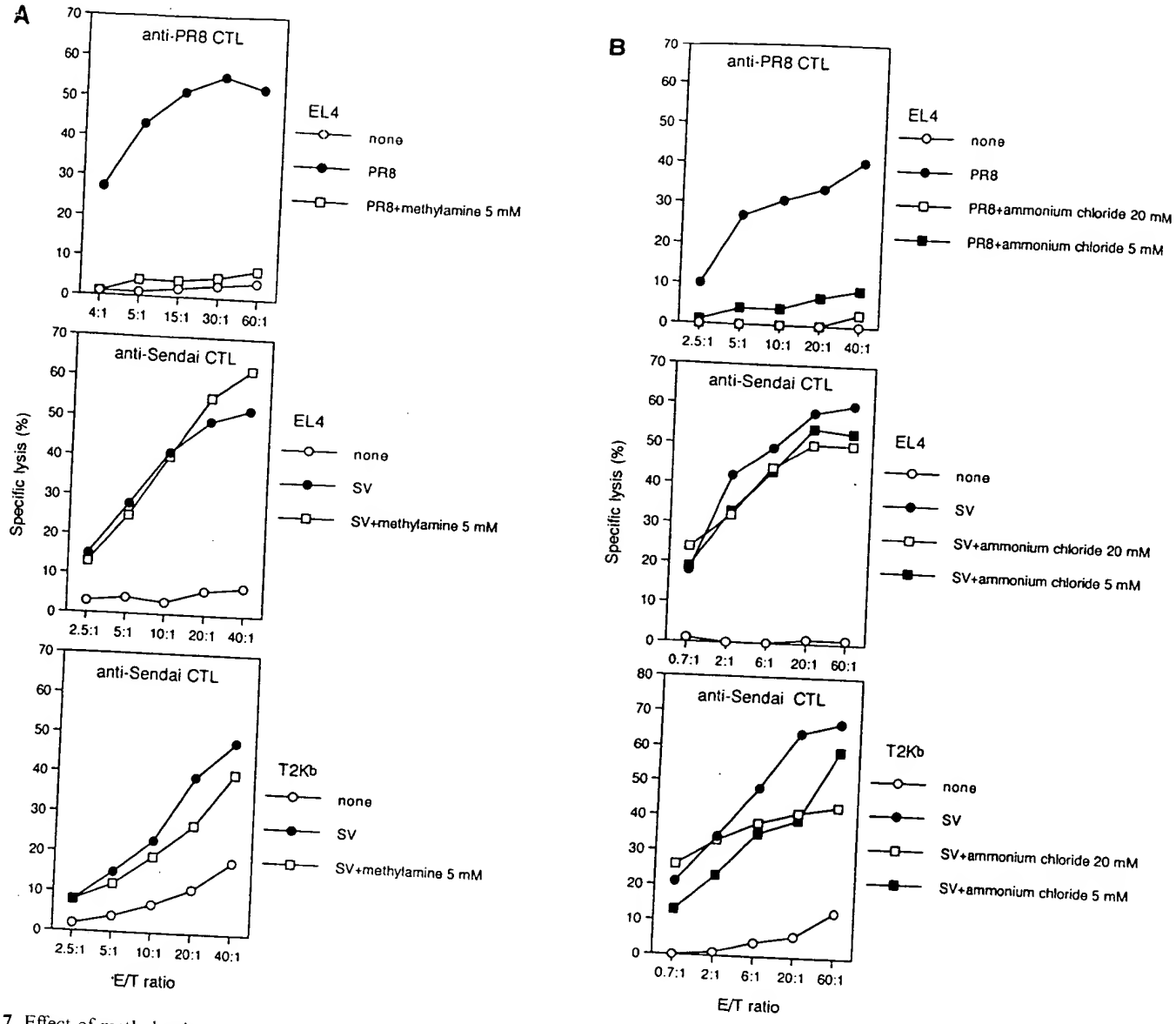

Fig. 7. Effect of methylamine (A) and ammonium chloride (B) on the presentation of influenza PR8 or Sendai virus antigen in target cells. EL-4 and $T2K^b$ cells were untreated or pretreated with methylamine (5 mM) or ammonium chloride (20 and 5 mM) and then infected with PR8 (640 HAU) or Sendai virus (500 HAU) in the absence or presence of the drugs.

Fourth, after prolonged infection, $T2K^b$ cells efficiently presented the Sendai minigene epitope expressed in recombinant vaccinia virus and this presentation was blocked by BFA. Fifth, Sendai virus antigen was presented in $T2K^b$ cells even in the presence of endosomal/lysosomal inhibitors, but the latter had a weak inhibitory effect on the presentation of Sendai virus antigen.

We have seen that MoAb against fusion (F) and haemaglutinin-neuraminidase (HANA) glycoproteins reduced presentation of Sendai virus by approximately 50% (data not shown). We interpret these findings to show that Sendai virus, possibly due to its fusiogenic characteristics at a physiological pH, can be taken up by endocytosis and subsequently, can enter into the TAP1/2-independent MHC class I processing pathway in $T2K^b$ cells. Whether Sendai viral proteins produced in the cytosol also enter into this non-classical MHC class I processing pathway is presently not clear.

The presentation of Sendai virus antigen in the TAP-independent and the BFA-resistant manner was found only in the $T1K^b$, $T2K^b$ and $T2K^b$TAP1/2 cells which are derived from a hybrid between the B lymphoblastoid cell line 721.174 and the T cell line CEM®.3. It is possible that these hybrid cells contain a unique intracellular compartment for association of peptides with MHC class I molecules. It is possible also that the present pathway may exist and operate in certain cells during physiological conditions. Dendritic cells (DC) are the principal antigen-presenting cells *in vivo* for the generation of both $CD8^+$ CTL and $CD4^+$ T helper responses

[46, 47]. It may be of importance to investigate whether Sendai virus antigen can gain access to a BFA-resistant class I processing pathway in such cells, or in other cells such as macrophages.

Harding and co-workers recently have reported a novel vacuolar class I processing pathway [19]. An epitope derived from OVA restricted by H-2K^b was expressed as fusion protein in *Escherichia coli*. Macrophages that were fed with these bacteria were capable of presenting this hidden OVA epitope to CD8$^+$ T cells in a class I-restricted fashion. Treatment of macrophages with cycloheximide or Brefeldin A, which block the classical class I processing pathway, had no effect on H-2K^b-restricted presentation of the OVA fusion protein. Rock *et al.* have reported the presentation of exogenous OVA antigens by H-2K^b in a subset of macrophages and DC from mouse spleen [48]. They conclude that these macrophages may deliver antigens from the extracellular fluid into the cytosol as the MHC class I-restricted presentation of exogenous OVA is sensitive to Gelonin, a protein synthesis inhibitor [49] which inhibits class I *de novo* synthesis. This delivery of exogenous antigens into the cytosol in macrophages could reflect a specific transport mechanism.

With the vaccinia minigene construct, we found that prolongation of infection of T2K^b cells with the vaccinia minigene epitope resulted in the TAP-independent and BFA-sensitive presentation of the Sendai minigene epitope. This suggested that a high concentration of cytosolic peptides can diffuse from the cytosol into the ER for loading onto class I molecules by overcoming the TAP defect. This is consistent with other studies using the stable episomal transfection and vaccinia expression systems by showing that some minigene epitopes and HIV-1 envelope protein could be presented to class I-restricted CD8$^+$ T cells, respectively [20, 21]. However, our results show further that the minigene-derived peptides may enter the exocytic MHC class I processing compartment, probably the ER, as BFA inhibits the presentation in T2K^b cells.

It has been demonstrated that the lysosomal inhibitors inhibit MHC class II- restricted antigen presentation and in some cases, also class I-restricted presentation [41, 43, 45]. Indeed, conflicting results have been obtained using these weak base inhibitors. The effect of the weak base inhibitors varies according to the cellular origin of the antigen-presenting cells, the nature of the T-cell epitope, and the responding T cell [45]. Clearly, these drugs block lysosomal/endosomal proteolysis by increasing the pH, thereby inhibiting the activity of acidic proteases present in this compartment (reviewed in [42]). The report described here showed that methylamine and ammonium chloride weakly inhibited the presentation of Sendai virus antigen in the mutant T2K^b cells as well as in non-mutant EL-4 cells. This could either reflect a minor role of the endosomal/lysosomal compartment in the TAP-independent MHC class I processing pathway or depend on incomplete inhibition by the drugs. It is known that a very small number of processed peptides (10–100) is needed to sensitize target cells for CTL killing [50]. As MHC class I molecules are recycled from the plasma membrane [51], the binding and processing of antigenic peptides might take place in the endosomal compartment. Furthermore, autophagosomes have the capacity to engulf cytosol and subsequently fuse with endosomes to redirect class I molecules and peptides to the endocytic route and hence to cell surface expression [23].

The study described also showed that both the BFA-resistant and BFA-sensitive pathways exist in cells of the T1 lineage despite the presence or absence of the TAP proteins, and that these pathways are accessible, depending on the virus in which the epitope is contained. If the epitope is part of the Sendai virus, it enters the BFA-resistant pathway. If the epitope is delivered by recombinant vaccinia virus as a minigene, it follows the BFA-sensitive ER-dependent MHC class I processing pathway. This may have some implications for the design of vaccines in order to target antigenic epitopes either into the BFA-sensitive or the BFA-resistant MHC class I-restricted antigen presentation pathways.

Finally, here we propose hypothetical models for the TAP-independent presentation of Sendai virus antigen by MHC class I molecules (Fig. 8). The live Sendai virus fuses with the plasma membrane (PM) and the nucleocapsid core is introduced into the cytosol. Viral proteins are then produced and degraded by the proteasomes into peptides that may enter the autophagosomes (I), formed by invagination of the cytosol by membranes from the ER. These autophagosomes may fuse with different compartments including the endosomes and the trans-Golgi network (TGN), where peptides bind 'empty' MHC class I molecules. Alternatively, viral proteins may be

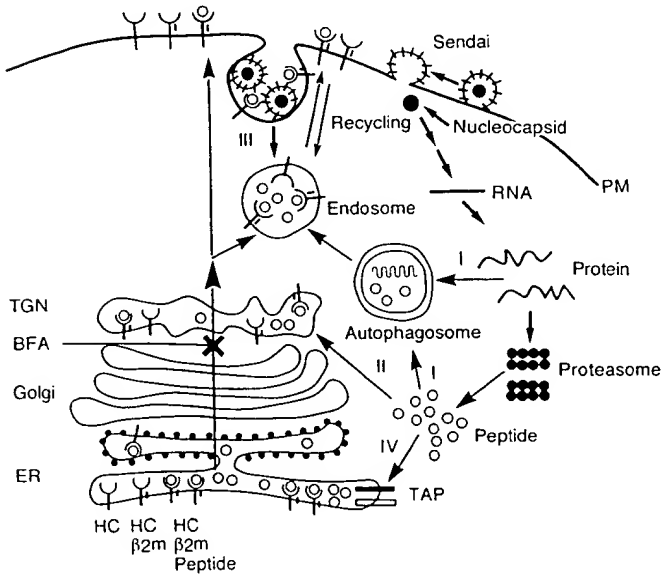

Fig. 8. Hypothetical models for TAP-independent and BFA-resistant presentation of Sendai virus antigen in T2K^b cells. For explanation see Discussion.

proteolytically processed in the autophagosomes (I). Peptides in the cytosol may also gain access to the TGN (II). Heat-inactivated or denatured Sendai virus might be taken up by endocytosis and be processed in the endosomes where peptides meet with 'empty' class I molecules delivered either from the ER or recycled from the cell surface (III). The endocytosed Sendai antigen may also be processed in the cytosol. In the case of the epitope delivered by recombinant vaccinia virus, the cytosolic epitope may diffuse into the lumen of the ER by overcoming the TAP deficiency (IV) and further transport of the peptide-class I complexes can be blocked by BFA. All pathways peripheral to the Golgi system are operational in BFA treated cells and TAP-independent, and could thus be considered for processing of Sendai virus antigen in $T2K^b$ cells.

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Characterization of TAP-Independent and Brefeldin A-Resistant Presentation of Sendai Virus Antigen to CD8$^+$ Cytotoxic T Lymphocytes

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Zhou X, Liu T, Franksson L, Lederer E, Ljunggren H-G, Jondal M. Characterization of TAP-Independent and Brefeldin A-Resistant Presentation of Sendai Virus Antigen to CD8$^+$ Cytotoxic T Lymphocytes. Scand J Immunol 1995;42:66–75

H-2K^b-transfected T2 cells, which lack both TAP1/2 and LMP2/7 genes, are able to efficiently process and present Sendai virus Antigen to K^b-restricted Sendai virus-specific CTL. This presentation is not inhibited by Brefeldin A (BFA). Here we extend our analysis of this novel antigen presentation pathway. We show that presentation of Sendai virus antigen was not due to sensitization of T2K^b cells by peptides in the virus preparation or peptides released from virus infected cells. Also, the ability to present Sendai virus in a BFA resistant fashion was specific for cells of the T2 lineage. Re-expression of TAP1/2 genes in T2K^b cells did not alter the capability to present antigen in a BFA resistant fashion, i.e. the presence of a functional TAP transporter complex did not relocate (all) peptides to the classical pathway for antigen processing and presentation. We found that co-infection of T2K^b cells with either Sendai virus plus influenza virus or Sendai virus plus VSV did not relocate presentation of influenza or VSV antigen to the TAP independent BFA resistant antigen presentation pathway. Peptide elution experiments and studies with peptide-specific CTL firmly demonstrated that the antigen presented by T2K^b cells after infection with Sendai virus was the natural Sendai virus epitope NP324-332. The same epitope, when expressed as a minigene in vaccinia virus, could be presented also by T2K^b cells but this presentation could be blocked by BFA. Thus, the TAP independent BFA resistant presentation of antigen seem cell (T2 lineage) and virus (Sendai virus) specific, but not epitope specific. The ability of T2K^b cells to present Sendai virus antigen in a TAP independent BFA resistant fashion was only partially blocked by lysosomal inhibitors such as methylamine, ammonium chloride and chloroquine. These findings demonstrate that TAP1/2-independent and BFA-resistant class I processing is only expressed in certain cell types, in parallel with classical MHC class I processing, and that Sendai virus selectively can enter this pathway. Hypothetical models for the TAP-independent class I processing are discussed.

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INTRODUCTION

CD8$^+$ cytotoxic T lymphocytes (CTL) kill virus-infected cells by recognizing processed viral peptides in association with MHC class I molecules on the surface of infected cells [1, 2].

Abbreviations: SV, Sendai virus; VSV, vesicular stomatitis virus; PR8, influenza virus A/Puerto Rico/8/34; NP, nucleoprotein; SV9, Sendai nucleoprotein peptide NP324-332; OVA, ovalbumin; VSV8, VSV nucleoprotein peptide NP52-57; BFA, Brefeldin A; VV-SNP$_{324-332}$, recombinant vaccinia virus encoding Sendai NP325-332; DC, dendritic cells; TAP, transporters associated with antigen processing; LMP, low molecular weight polypeptides.

Viral antigens undergo a limited degradation in which the multicatalytic proteinase complex, or proteasome, may be involved (reviewed in [3]). The resulting peptides are delivered to the lumen of the ER by the help of TAP proteins [4–9]. The class I-peptide complexes are then transported to the cell surface for recognition by CTL [1, 2].

The TAP complex consists of two subunits, TAP1 and TAP2, forming a heterodimer in the membrane of the ER and the cis-Golgi [10–13]. The genes of TAP1 and TAP2 are localized in the class II region of the MHC [14–17] (reviewed in [18]). More recently, it has been demonstrated directly that

the TAP proteins are important in transport of cytosolic peptides into the ER in an ATP-dependent and peptide-selective manner [4–9]. Human TAP and rat TAP^a translocate peptides with hydrophobic and basic C termini, whereas mouse TAP and rat TAP^a selectively transport peptides with hydrophobic C termini with a usual size of 8–14 residues [8, 9].

Several reports have challenged the absolute requirement for the TAP proteins in class I-restricted antigen presentation [19–22] (reviewed in [23]). Peptide presentation restricted by class I molecules apparently can be achieved in macrophages after phagocytosis of recombinant bacteria containing an ovalbumin (OVA) fusion protein [19] as well as in TAP-deficient T2 cells after peptide minigene transfection [20] or HIV-1 envelop protein uptake [21]. We have observed previously that the antigen processing mutant T2 cells, which lack both TAP1/2 peptide transporters and LMP2/7 proteasome subunits, and thus are unable to present endogenously synthesized antigens [24–26], are killed readily by Sendai-specific CD8$^+$ CTL after K^b transfection and viral infection [22]. Intriguingly, the presentation of Sendai virus antigen in T2K^b cells is not blocked by BFA, as was also reported for OVA recombinant *Escherichia coli* or *Salmonella typhimurium* bacteria [19], indicating the presence of an alternate pathway for presentation by class I molecules.

To characterize further the TAP-independent and BFA-resistant presentation of Sendai virus antigen, we have analysed the presentation in different cell types, the effect of endosomal/lysosomal inhibitors, and the influence of Sendai virus on presentation of influenza virus and VSV antigens. We have investigated also whether an immunodominant Sendai epitope (NP324-332) is processed in T2K^b cells and whether the same epitope, if expressed as a vaccinia-based minigene, is presented in the BFA-sensitive or -resistant pathways.

MATERIALS AND METHODS

Cell lines and culture conditions. EL-4 (H-2^b) is a thymoma cell line derived from C57BL/6 mice. P815K^b, HYM2K^b and JurkatK^b cells are: P815 (mastocytoma of DBA/2, H-2^d), HYM2 (EBV-transformed human B lymphoma) and Jurkat (human T lymphoma) cell lines transfected with H-2K^b genes, respectively. T1 is a hybrid of the B lymphoblastoid cell line 721.174 and the T cell line CEM®.3. T2 is selected from T1 cells for loss of CEM®.3-derived chromosome 6 and has a large deletion in the MHC class II region including genes for TAP1/2 and LMP2/7 [27, 28]. T1K^b, T2K^b and T2K^{b}TAP1/2 (T2K^b cells transfected with rat TAP1/2) [29] are T1, T2 and T2TAP1/2 cells transfected with H-2K^b genes. P815K^b was a gift from Dr H. Stauss (University College, London). JurkatK^b was kindly provided by Dr L. A. Sherman (The Scripps Research Institute, La Jolla, CA, USA) via Dr H. N. Eisen (MIT, Cambridge, MA, USA). HYM2K^b, T1K^b, T2K^b and T2K^{b}TAP1/2 transfectants were generated by Drs F. Momburg and G. J. Hämmerling (German Cancer Research Center, Heidelberg, Germany) and characteristics of T1K^b, T2K^b and T2K^{b}TAP1/2 have been described [30]. EL-4 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, penicillin and streptomycin. All transfectants were cultivated in RPMI 1640–10% FCS medium and Geneticin (G-418 Sulfate, 0.5 mg/ml) (Gibco, BRL, UK). Expression of transfected MHC class I genes were regularly checked by FACS analysis and virus-specific CTL assay.

Mice, viruses and synthetic peptides. C57BL/6 (H-2^b) and BALB/c (H-2^d) female mice were purchased from B & K Universal AB (Stockholm, Sweden). All mice were used at the age of 6–10 weeks. Non-virulent Sendai virus, lot 40340087, was obtained originally from Flow Laboratories and was generously provided by Drs W. M. Kast and C. J. M. Melief (University Hospital, Leiden, The Netherlands). Influenza virus A/Puerto Rico/8/34 (PR8) was a gift from Dr A. Douglas (National Institute for Medical Research, London, UK). The Sendai virus and the influenza virus were grown in the allantoic cavity of embryonated chicken eggs and used as allantoic fluid for priming and infecting target cells. VSV Indiana serotype was a gift from Dr K. Kristensson (Huddinge University Hospital, Karolinska Institute, Stockholm, Sweden). The VSV was grown in Vero cells and plaqued on BHK-21 cells. The recombinant vaccinia virus encoding NP325-332 (VV-SNP$_{M325-332}$) ([31] it had an error in sequence numbering VV-SNP$_{M321-328}$) (*M*APGNYPAL, a methionine for initiation and also for substitution of phenylalanine) was generously provided by Dr J. R. Bennink (National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA). The VV-SNP$_{M325-332}$ was grown and plaqued in BHK-21 cells. The Sendai nucleoprotein (NP) peptide 324-332 (FAPGNYPAL, K^b-binding, SV9) [32, 33], VSV nucleocapsid (N) peptide 52-57 (RGYVYQGL, K^b-binding, VSV8) [34] and influenza NP peptide 147-155 (TYQRTRALV, K^d-binding, influenza NP147-155) [35] were synthesized by Drs B. R. Srinivasa (Astra Research Center, Bangalore, India) and U. Rudén (SMI, Stockholm) using f-moc synthesis on a peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA, USA) and the manual 'tea-bag' solid phase method. Peptides were purified by reverse phase HPLC. Stock solution of peptides were prepared in PBS and stored at −20°C.

Isolation of antigenic peptides by affinity purification and HPLC fractionation. T2K^b cells (5 × 10^8) were infected with 40 ml Sendai virus (2560 HAU/ml) for 1.5 h. After washing, cells were incubated for 1.5 h for monitoring infection by CTL assay or 7.5 h for isolation of peptides. After completion of incubation, cells were washed three times with PBS and frozen at −20°C as a pellet. The same amount of uninfected cells were used as control. Affinity purification of MHC class I molecules and HPLC fractionation of this material were done as described [36]. Briefly, infected and non-infected T2K^b cells were lysed by stirring the cells for 60 min in 20 ml PBS containing 0.5% NP40. The lysates were centrifuged at 23,400 *g* for 30 min using a Sorvall RC-5C centrifuge and a SS34 rotor (Sorvall Instruments, Du Pont, Instrument AB Lambada, Stockholm, Sweden). The pH of the supernatants was adjusted to 8.0 by adding 1 M Tris-HCl buffer pH 8.0. The lysates were pre-cleared by running through columns with Pharmacia 4CL-protein A-Sepharose beads loaded with antibodies from normal mouse serum. In a second step, the pre-cleared lysates were run through an immunoaffinity column specific for H-2K^b (4CL-protein A-Sepharose beads loaded with AF6-88.5.3, K^b α1/α2 specific MoAb). Prior to elution of the bound MHC class I molecules, the columns were washed with 10 mM Tris-HCl buffer pH 8.0. Loaded beads were subject to acid extraction using 100 mM glycin pH 3.0. Bed volumes, 2 ml; flow rates, 0.5 ml/min. Trifluoroacetic acid (TFA) was added to the collected material to a final concentration of 0.1%, which then was incubated for 15 min swirling. All work was performed at 4°C. The collected material was separated on HPLC.

Separation by reverse phase HPLC. The separations were performed on a reverse phase Superpac Pep S column (C2/C18, 5 mM particles, 4.0 × 250 mm; Pharmacia LKB, Uppsala, Sweden) using Pharmacia LKB equipment. Elution procedures: solution A, 0.1% TFA in H_2O; solution B, 0.1% TFA in acetonitrile; 0–5 min, 100% A; 5–45 min, linear increase to 60% B; 45-50 min, 60% B; 50-55 min, linear decrease to 0% B. Flow rate 1 ml/min. Elution was monitored by measuring UV-light absorption at 214 nm in a continuous flow detector, and 1 ml fractions were collected. Individual fractions were dried by vacuum centrifugation.

Generation of virus- and peptide-specific CTL. Generation of virus-specific CTL was done as described [25, 37]. Mice were immunized i.v. or i.p. with 20–100 HAU of Sendai virus or PR8 or i.p. with 10^6 PFU of VSV. After 1–2 weeks, immune spleen cells (25×10^6) were restimulated with virus-infected syngeneic spleen cells (2000 rad, 25×10^6) in 50-ml tissue culture flasks (Costar, Cambridge, MA, USA) with 15 ml RPMI 1640 complete medium (supplemented with 10% FCS, L-glutamine, non-essential amino acids, sodium pyruvate and antibiotics) for 5 days at 37°C, 5% CO_2. The optimal conditions for generation of peptide-specific CTL have been described in detail [37, 38]. Mice were immunized s.c. with 100 mg peptide emulsified in incomplete Freund's adjuvant (IFA). After 7–10 days, immune spleen cells were restimulated with irradiated syngeneic spleen cells in the presence of low concentration of peptide (0.05 mM) for 5 days.

CTL assay. A standard ^{51}Cr-release assay was performed. Preparation of target cells were done by infecting cells ($1–2 \times 10^6$) with virus or incubation with peptides (50 μM) for 1.5 h at 37°C. After virus infection, cells were washed and further incubated for 2–4 h. Target cells were labelled with ^{51}Cr (100 mCi, Dupont, Boston, MA, USA) for 1 h, washed, adjusted to the appropriate concentration and mixed with titrated effector CTL in V-bottomed 96-well plates and incubated for 4–6 h at 37°C. Supernatants were collected and radioactivity measured in a gamma-counter. Results are expressed as percentage of specific lysis according to the formula: % Specific lysis = [(experimental release-spontaneous release) / (maximal release-spontaneous release)] cpm × 100. Vacuum-dried HPLC fractions were dissolved in 200 μl of distilled water and aliquots of 10 μl were plated into V-bottomed 96-well plates. ^{51}Cr-labelled target cells (1×10^4) were added to each well for 30 min incubation at 37°C, and followed by addition of effector CTL. All CTL experiments were reproduced at least three times and representative experiments are shown in the Results section.

Inhibitor treatment. Brefeldin A (BFA) was kindly provided by Sandoz AB (Täby, Sweden), dissolved in methanol (10 mg/ml) and stored at −20°C. Methylamine, ammonium chloride and chloroquine were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in RPMI 1640 medium. Treatment of target cells with BFA or lysosomotropic agents were described [39–45]. Cells were pretreated with BFA (10 mg/ml) or methylamine (5 mM) or ammonium chloride (20 and 5 mM) for 30 min and infected with virus or incubated with peptide for 1.5 h in the presence or absence of inhibitors. After washing, cells were further incubated for 2–4 h with or without inhibitors before labelling with ^{51}Cr and the CTL assay. The inhibitors were present in the CTL assay.

RESULTS

BFA-resistant presentation of Sendai virus antigen in T2K^b cells

We have found previously that Sendai virus antigen can be processed in T2K^b cells for K^b presentation and that this function is resistant to BFA ([22] and Fig. 1). The possibility that T2K^b cells might be sensitized by contaminating peptide in the Sendai virus preparation has been excluded by the separation of the virus preparation into a low molecular weight fraction (Table 1). No target cell sensitization was seen using the low molecular weight (below 10 kDa) fraction. Furthermore, T2K^b cells did not release K^b-binding peptides after Sendai virus infection as no killing of uninfected ^{51}Cr-labelled by-stander T2K^b cells was seen (Table 2).

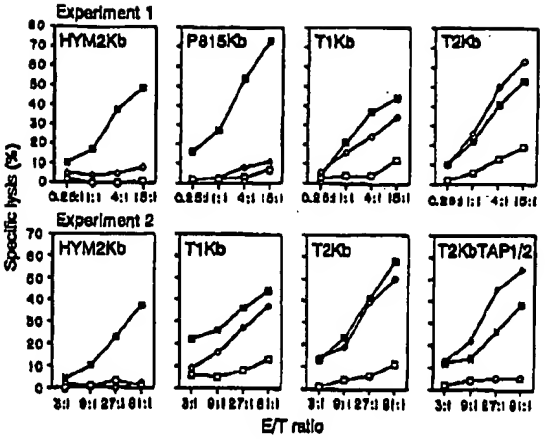

Fig. 1. BFA-sensitive and -resistant presentation of Sendai virus antigen in different cell lines. HYM2K^b, P815K^b, T1K^b, T2K^b and T2K^{b}TAP1/2 cells were left untreated, infected with Sendai virus alone (500 HAU), or with virus in the presence of BFA and assayed for specific CTL lysis. –□–, none; –■–, SV; –◇–, SV + BFA.

Table 1. No peptide contamination in the Sendai virus preparation

Target cells	Treatment[a]	CTL killing[b] 30:1	10:1	3:1
EL-4	untreated	2	1	1
	SV	49	23	12
	SV-filtered	2	1	1
T2K^b	untreated	8	6	4
	SV	49	27	18
	SV-filtered	5	3	2

[a] Target cells were infected with Sendai virus (SV) (250 HAU) or the low mw (10 kDa cut-off) fraction of filtered SV and tested for CTL lysis. To generate the low mw fraction, Sendai virus (corresponding to 250 HAU) was loaded on a 10 kDa cut-off membrane separator and centrifuged at 4300 *g* for 3 h at 4°C.

[b] Per cent specific lysis at the respective E:T ratios indicated.

Table 2. No release of peptide from Sendai virus-infected $T2K^b$ cells

Target cells	CTL killing[a] 27:1	9:1	3:1
^{51}Cr-$T2K^b$	8	2	2
^{51}Cr-$T2K^b$-SV	73	63	49
^{51}Cr-$T2K^b$-SV9	84	82	70
$T2K^b$-SV(cold) + ^{51}Cr-$T2K^b$(hot)	4	2	0

[a] Per cent specific lysis at the respective E:T ratios indicated.

BFA-sensitive and -resistant presentation of Sendai virus antigen in different cell lines

Different cell lines were tested for BFA-sensitive and resistant Sendai virus antigen processing and presentation to Sendai virus specific CTL (Fig. 1). The presentation in K^b-transfected HYM2 (B-LCL) and P815 (mastocytoma) cells was inhibited by BFA, suggesting that only the classical MHC class I processing pathway was operative in these cells. In contrast, presentation of Sendai virus antigen cannot be blocked by BFA in either $T1K^b$, $T2K^b$ or $T2TAP1/2K^b$ cells. Since $T1K^b$ as well as $T2TAP1/2K^b$ cells express TAP1/2 peptide transporters, and can present endogenous antigen via a functional classical MHC class I processing pathway [30], these results demonstrate that the non-classical, BFA-resistant MHC class I processing occurs also in these parental cells and that these two pathways can operate in parallel. Possibly, there is a partial inhibition of Sendai virus antigen processing by BFA in $T1K^b$, which may not be seen in TAP1/2-transfected $T2K^b$ cells (Fig. 1). Nonetheless, the data suggest that different cell types can express different MHC class I processing pathways either selectively or in parallel.

Co-infection of Sendai virus with VSV or influenza virus

In order to assess whether the presence of Sendai virus could induce processing and presentation of VSV or PR8 derived viral antigens in T2 cells (neither of these viruses are presented in $T2K^b$ or $T2K^d$ cells, respectively, [22, 25, 30], we infected $T2K^b$ or $T2K^d$ cells with VSV or PR8 virus alone or co-infected these with titrated doses of Sendai virus. No presentation of VSV or PR8 antigens in $T2K^b$ or $T2K^d$ cells was observed, even if these viruses were co-infected with 1000 HAU of Sendai virus. As expected, control $T1K^b$, $T1K^d$ or P815 cells efficiently presented endogenously synthesized VSV or PR8 antigens (Fig. 2, data not shown). In conclusion, the presence of Sendai virus was not sufficient to re-target presentation of VSV and influenza antigens into the TAP-independent class I processing pathway.

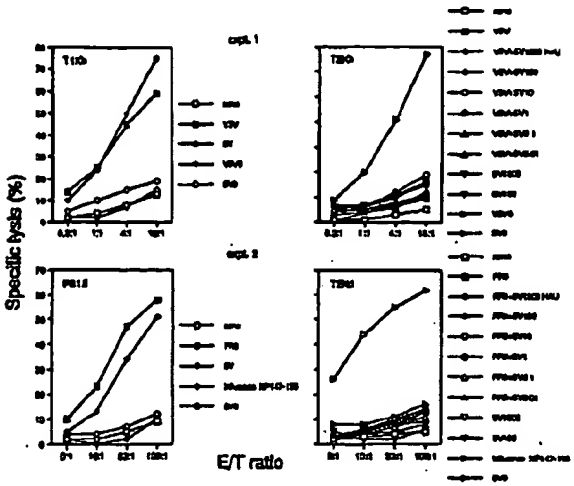

Fig. 2. Co-infection of Sendai virus with VSV or influenza PR8 in $T2K^b$ or $T2K^d$ cells. Expt. 1: $T1K^b$ and $T2K^b$ cells were untreated, infected with VSV (2 PFU), coinfected with VSV (2 PFU) and titrated doses of Sendai virus ranging from 1000 to 0.01 HAU or incubated with the VSV8 peptide, and tested for cytolysis by VSV CTL. Expt. 2: P815 and $T2K^d$ were untreated, infected with PR8 alone (640 HAU), co-infected with PR8 and titrated concentrations of Sendai virus or incubated with the influenza peptide NP147-155, and assayed for anti-PR8 CTL killing.

CTL recognition of HPLC fractions from Sendai virus-infected $T2K^b$ cells

In order to identify the Sendai virus epitope presented by $T2K^b$ cells after infection with Sendai virus, $T2K^b$ cells were infected with Sendai virus and H-$2K^b$ molecules were affinity-purified. After acidic elution, the peptide materials were further separated by reverse phase HPLC and 40 fractions

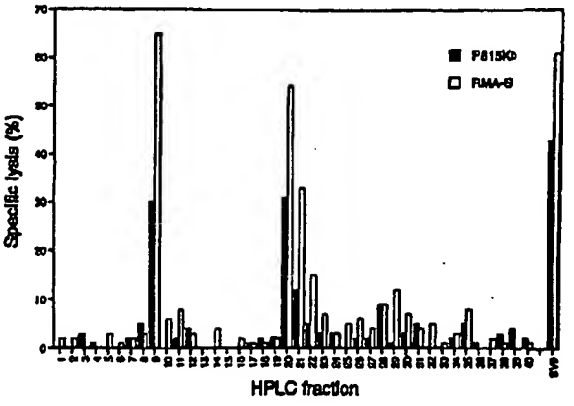

Fig. 3. Recognition of two major HPLC fractions eluted from Sendai virus-infected $T2K^b$ cells by Sendai virus-specific CTL. Target cells were $P815K^b$ and RMA-S. A similar profile was also seen in $T2K^b$ target cells (data not shown). E/T ratio, 50:1.

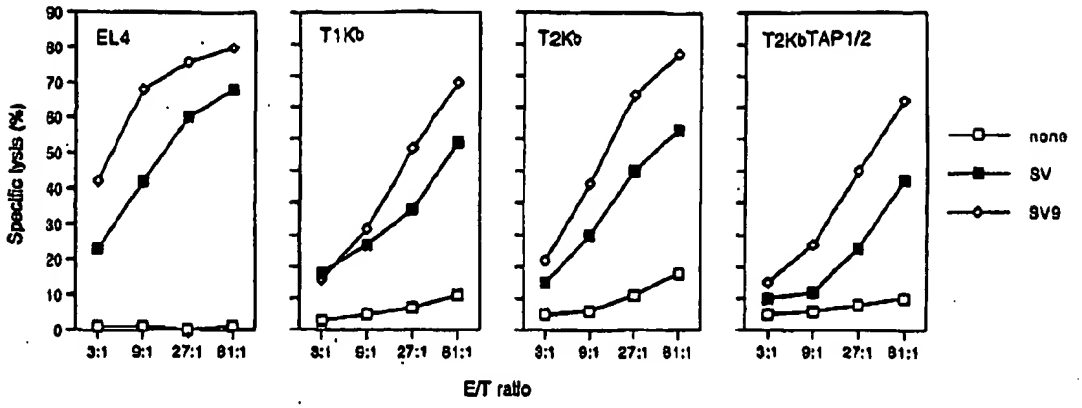

Fig. 4. Recognition of Sendai virus-infected T2K^b cells by Sendai peptide-specific CTL. Peptide-peptide CTL, primed with peptide *in vivo* and restimulated with peptide *in vitro*. Sendai virus-peptide CTL (primed with Sendai virus and restimulated with peptide) were used as control (data not shown).

were collected. Two active fractions (Fig. 3, No.9 and 20) were found strongly to sensitize P815K^b, RMA-S as well as T2K^b (data not shown) target cells for Sendai virus-specific CTL. The synthetic SV9 peptide (NP325-332) eluted was close to fraction 20. This suggested that the Sendai virus antigen presented by H-2K^b in T2 cells, as expected, was of peptide nature and resembled the natural Sendai NP epitope presented by non-mutant H-2K^b expressing cells after Sendai virus infection.

Recognition of Sendai virus-infected T2K^b cells by peptide-specific CTL

Recently, we have defined optimal conditions for generation of CTL responses with short synthetic peptides. Such peptide-induced CTL show a high specificity of target cell recognition and exert a strong killing activity against virus-infected target cells [37, 38]. This allowed us to generate CTL specific for the Sendai virus peptides NP324-332. Such CTL efficiently killed T2K^b as well as control cells infected with Sendai virus (Fig. 4), demonstrating directly that T2K^b cells could process the Sendai virus NP324-332 antigen for presentation to peptide specific CTL.

Presentation of the Sendai minigene epitope in T2K^b cells

EL-4 (H-2^b), JurkatK^b and T2K^b target cells were infected for a short (3 h) or long (12 h) time with recombinant vaccinia virus encoding Sendai NP325-332 (VV-SNP$_{M325-332}$) and tested for their antigen presenting ability to CTL. Figure 5

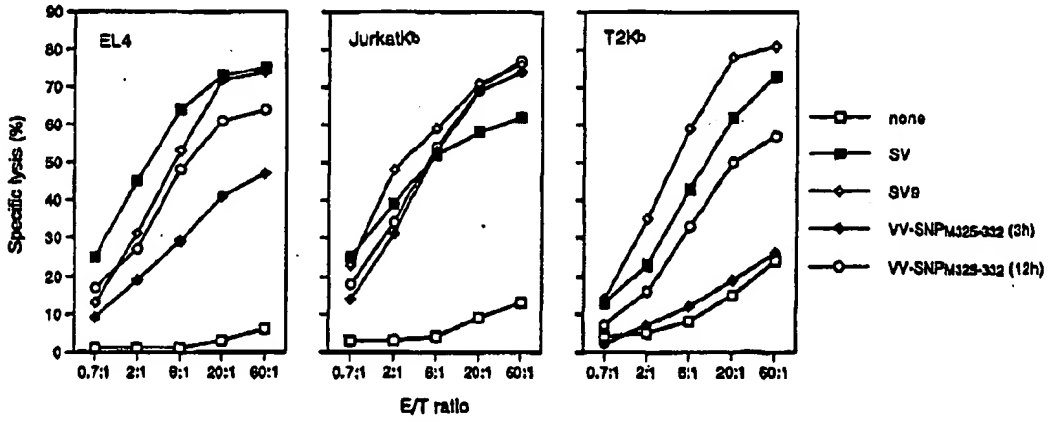

Fig. 5. T2K^b cells presented the minigene-derived peptides after prolongation of infection. EL-4, JurkatK^b and T2K^b cells were untreated, infected with Sendai virus (500 HAU) or VV-SNP$_{M325-332}$ (200 PFU, 3 h or 12 h), or incubated with SV9 and tested for CTL killing. Target cells infected with the vaccinia vector alone were not killed (data not shown).

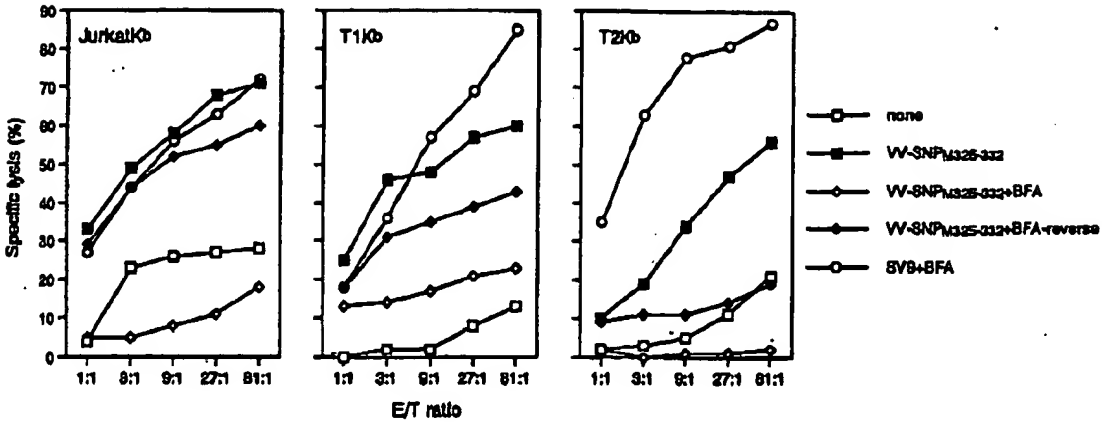

Fig. 6. BFA blocked the presentation of the endogenously minigene-derived peptides in T2K^b cells. JurkatK^b, T1K^b and T2K^b cells were untreated, infected with VV-SNP$_{M325-332}$ (12 h) or incubated with SV9 in the absence or presence of BFA. The BFA blocking effect was reversible. Target cells infected with the vaccinia vector alone were not killed (data not shown).

shows that T2K^b cells after infection with VV-SNP$_{M325-332}$ for 3 h were unable to present the endogenous minigene epitope to Sendai virus-specific CTL. However, after prolongation of infection to 12 h, a significant cytolysis of T2K^b cells was observed. Control EL-4 and JurkatK^b cells, with normal TAP expression, could efficiently present the minigene peptide even after a short time of infection. To exclude that a long time (12 h) of viral infection may lead to a cytopathic effect and thus release a peptide that contributes to sensitize innocent target cells, cell viability was checked. After infection with VV-SNP$_{M325-332}$ for 12 h the cell viability was not affected as determined by trypan blue staining (data not shown). Furthermore, the supernatants from VV-SNP$_{M325-332}$-infected (12 h) T2K^b cells did not sensitize target cells for lysis by Sendai virus CTL (data not shown). Thus, T2K^b cells, after prolonged infection, could present the Sendai minigene epitope independent of TAP expression.

Presentation of the Sendai minigene epitope in T2K^b cells was blocked by BFA

To understand whether the Sendai minigene epitope follows a similar BFA-resistant presentation pathway as Sendai virus in T2K^b cells, we assessed the effect of BFA on the presentation of the minigene epitope (Fig. 6). T2K^b cells as well as the wild-type T1K^b cells presented the Sendai minigene epitope NP325-332. For both cells, this presentation could be blocked by BFA (Fig. 6). This result suggested that the TAP-independent presentation of the Sendai minigene epitope in T2K^b cells could occur, but that it was possible to block this presentation with BFA. Thus, the presentation of the Sendai virus epitope NP325-332 follows two different pathways dependent on where the epitope is expressed.

Effect of lysosomal inhibitors on the presentation of Sendai virus antigen in T2K^b cells

To study whether the endosomal/lysosomal compartments with low pH were involved in processing of Sendai virus antigen, methylamine, ammonium chloride and chloroquine were used. Rock and colleagues have described that methylamine and ammonium chloride inhibits class I- and class II-restricted antigen presentation, and in contrast, that chloroquine markedly inhibited class II but not class I-restricted antigen presentation [41]. Figure 7A and B showed that methylamine and ammonium chloride had a weak inhibitory effect on the presentation of Sendai virus antigen in T2K^b as well as in EL-4 cells. As expected, these drugs strongly inhibited the presentation of influenza virus antigen in EL-4 cells as endosomal fusion steps, which are necessary for influenza virus infection, are affected by these drugs (Fig. 7A and B [44]). The concentrations of the drugs used in the present study showed no toxicity in the course of the assay on effector cells as target cells were killed in the presence of exogenously provided peptides (data not shown).

DISCUSSION

In the study described here we made the following observations. First, the TAP-independent and BFA-resistant presentation of Sendai virus antigen was detected only in the T1K^b, T2K^b and T2K^{b}TAP1/2 cells. Second, the presence of Sendai virus was not sufficient to redirect VSV and influenza virus antigen into the TAP-independent and BFA resistant class I processing pathway. Third, the Sendai virus epitope presented by H-2K^b in T2 cells appear to be identical to the epitope NP325-332 presented by non-mutant cells of the H-2^b haplo-type; CTL recognized Sendai virus-infected T2K^b cells.

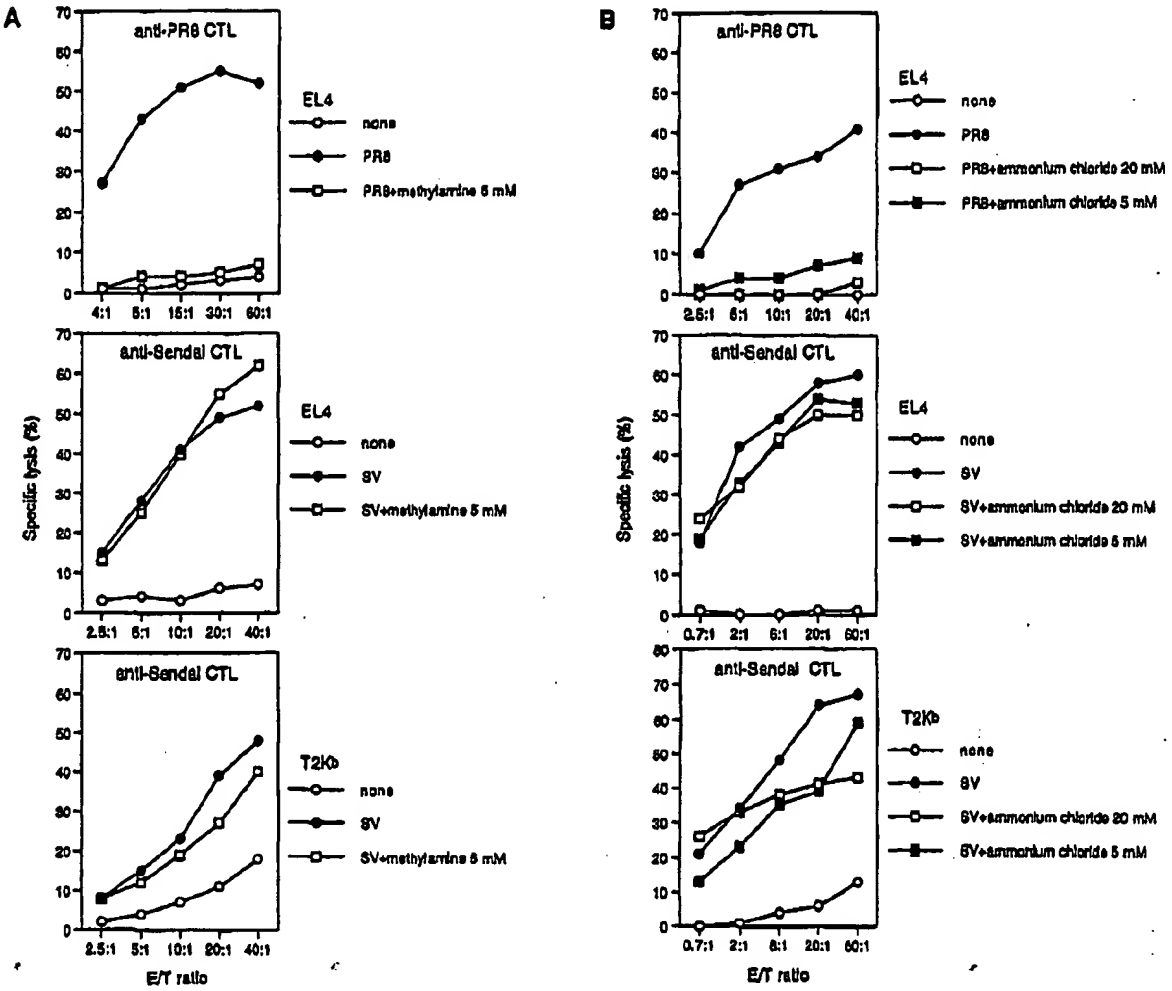

Fig. 7. Effect of methylamine (A) and ammonium chloride (B) on the presentation of influenza PR8 or Sendai virus antigen in target cells. EL-4 and T2K^b cells were untreated or pretreated with methylamine (5 mM) or ammonium chloride (20 and 5 mM) and then infected with PR8 (640 HAU) or Sendai virus (500 HAU) in the absence or presence of the drugs.

Fourth, after prolonged infection, T2K^b cells efficiently presented the Sendai minigene epitope expressed in recombinant vaccinia virus and this presentation was blocked by BFA. Fifth, Sendai virus antigen was presented in T2K^b cells even in the presence of endosomal/lysosomal inhibitors, but the latter had a weak inhibitory effect on the presentation of Sendai virus antigen.

We have seen that MoAb against fusion (F) and haemaglutinin-neuraminidase (HANA) glycoproteins reduced presentation of Sendai virus by approximately 50% (data not shown). We interpret these findings to show that Sendai virus, possibly due to its fusiogenic characteristics at a physiological pH, can be taken up by endocytosis and subsequently, can enter into the TAP1/2-independent MHC class I processing pathway in T2K^b cells. Whether Sendai viral proteins produced in the cytosol also enter into this non-classical MHC class I processing pathway is presently not clear.

The presentation of Sendai virus antigen in the TAP-independent and the BFA-resistant manner was found only in the T1K^b, T2K^b and T2K^{b}TAP1/2 cells which are derived from a hybrid between the B lymphoblastoid cell line 721.174 and the T cell line CEM$^{\oplus}$.3. It is possible that these hybrid cells contain a unique intracellular compartment for association of peptides with MHC class I molecules. It is possible also that the present pathway may exist and operate in certain cells during physiological conditions. Dendritic cells (DC) are the principal antigen-presenting cells *in vivo* for the generation of both CD8$^+$ CTL and CD4$^+$ T helper responses

[46, 47]. It may be of importance to investigate whether Sendai virus antigen can gain access to a BFA-resistant class I processing pathway in such cells, or in other cells such as macrophages.

Harding and co-workers recently have reported a novel vacuolar class I processing pathway [19]. An epitope derived from OVA restricted by H-2K^b was expressed as fusion protein in *Escherichia coli*. Macrophages that were fed with these bacteria were capable of presenting this hidden OVA epitope to CD8$^+$ T cells in a class I-restricted fashion. Treatment of macrophages with cycloheximide or Brefeldin A, which block the classical class I processing pathway, had no effect on H-2K^b-restricted presentation of the OVA fusion protein. Rock *et al.* have reported the presentation of exogenous OVA antigens by H-2K^b in a subset of macrophages and DC from mouse spleen [48]. They conclude that these macrophages may deliver antigens from the extracellular fluid into the cytosol as the MHC class I-restricted presentation of exogenous OVA is sensitive to Gelonin, a protein synthesis inhibitor [49] which inhibits class I *de novo* synthesis. This delivery of exogenous antigens into the cytosol in macrophages could reflect a specific transport mechanism.

With the vaccinia minigene construct, we found that prolongation of infection of T2K^b cells with the vaccinia minigene epitope resulted in the TAP-independent and BFA-sensitive presentation of the Sendai minigene epitope. This suggested that a high concentration of cytosolic peptides can diffuse from the cytosol into the ER for loading onto class I molecules by overcoming the TAP defect. This is consistent with other studies using the stable episomal transfection and vaccinia expression systems by showing that some minigene epitopes and HIV-1 envelope protein could be presented to class I-restricted CD8$^+$ T cells, respectively [20, 21]. However, our results show further that the minigene-derived peptides may enter the exocytic MHC class I processing compartment, probably the ER, as BFA inhibits the presentation in T2K^b cells.

It has been demonstrated that the lysosomal inhibitors inhibit MHC class II- restricted antigen presentation and in some cases, also class I-restricted presentation [41, 43, 45]. Indeed, conflicting results have been obtained using these weak base inhibitors. The effect of the weak base inhibitors varies according to the cellular origin of the antigen-presenting cells, the nature of the T-cell epitope, and the responding T cell [45]. Clearly, these drugs block lysosomal/endosomal proteolysis by increasing the pH, thereby inhibiting the activity of acidic proteases present in this compartment (reviewed in [42]). The report described here showed that methylamine and ammonium chloride weakly inhibited the presentation of Sendai virus antigen in the mutant T2K^b cells as well as in non-mutant EL-4 cells. This could either reflect a minor role of the endosomal/lysosomal compartment in the TAP-independent MHC class I processing pathway or depend on incomplete inhibition by the drugs. It is known that a very small number of processed peptides (10–100) is needed to sensitize target cells for CTL killing [50]. As MHC class I molecules are recycled from the plasma membrane [51], the binding and processing of antigenic peptides might take place in the endosomal compartment. Furthermore, autophagosomes have the capacity to engulf cytosol and subsequently fuse with endosomes to redirect class I molecules and peptides to the endocytic route and hence to cell surface expression [23].

The study described also showed that both the BFA-resistant and BFA-sensitive pathways exist in cells of the T1 lineage despite the presence or absence of the TAP proteins, and that these pathways are accessible, depending on the virus in which the epitope is contained. If the epitope is part of the Sendai virus, it enters the BFA-resistant pathway. If the epitope is delivered by recombinant vaccinia virus as a minigene, it follows the BFA-sensitive ER-dependent MHC class I processing pathway. This may have some implications for the design of vaccines in order to target antigenic epitopes either into the BFA-sensitive or the BFA-resistant MHC class I-restricted antigen presentation pathways.

Finally, here we propose hypothetical models for the TAP-independent presentation of Sendai virus antigen by MHC class I molecules (Fig. 8). The live Sendai virus fuses with the plasma membrane (PM) and the nucleocapsid core is introduced into the cytosol. Viral proteins are then produced and degraded by the proteasomes into peptides that may enter the autophagosomes (I), formed by invagination of the cytosol by membranes from the ER. These autophagosomes may fuse with different compartments including the endosomes and the trans-Golgi network (TGN), where peptides bind 'empty' MHC class I molecules. Alternatively, viral proteins may be

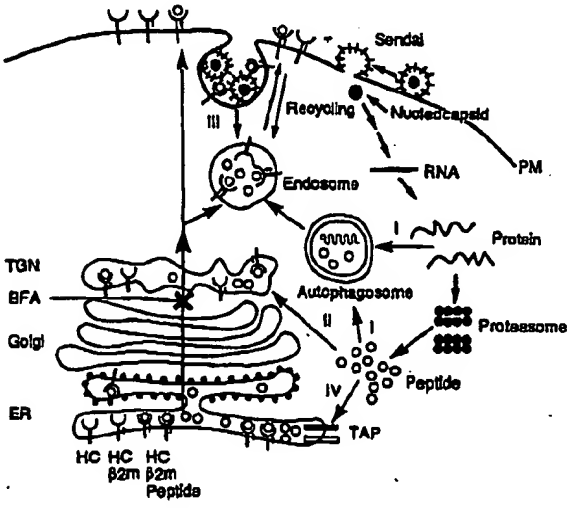

Fig. 8. Hypothetical models for TAP-independent and BFA-resistant presentation of Sendai virus antigen in T2K^b cells. For explanation see Discussion.

proteolytically processed in the autophagosomes (I). Peptides in the cytosol may also gain access to the TGN (II). Heat-inactivated or denatured Sendai virus might be taken up by endocytosis and be processed in the endosomes where peptides meet with 'empty' class I molecules delivered either from the ER or recycled from the cell surface (III). The endocytosed Sendai antigen may also be processed in the cytosol. In the case of the epitope delivered by recombinant vaccinia virus, the cytosolic epitope may diffuse into the lumen of the ER by overcoming the TAP deficiency (IV) and further transport of the peptide-class I complexes can be blocked by BFA. All pathways peripheral to the Golgi system are operational in BFA treated cells and TAP-independent, and could thus be considered for processing of Sendai virus antigen in $T2K^b$ cells.

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REVIEW
IMMUNOLOGY TODAY

TAP off – tumors on

Barbara Seliger, Markus J. Maeurer and Soldano Ferrone

The molecular characterization of T-cell-defined tumor-associated antigens has provided targets for cell-mediated immunotherapy for malignant diseases. The success of this strategy is negatively influenced by structural and functional abnormalities of major histocompatibility complex (MHC) class I molecules, which provide tumor cells with resistance to T-cell-mediated immune recognition. This article reviews the physiology of the MHC class I processing machinery and describes the deficiencies of this pathway in malignant cells.

In recent years, the molecular basis of the recognition of antigens by cytotoxic T lymphocytes (CTLs) has been elucidated. This information, in conjunction with significant progress in recombinant DNA technology and cellular immunology, has facilitated the identification of several human major histocompatibility complex (MHC) HLA class I-restricted tumor-associated antigens (TAAs) recognized by $CD8^+$ CTLs. The TAAs identified so far, and which are likely to represent only a small proportion of the large number of CTL-defined TAAs expressed by human tumor cells, can be segregated into three major groups: (1) TAAs encoded by normal, nonmutated genes that are expressed by tumor cells but are silent in normal cells, such as members of the MAGE family[1–5]; (2) tissue-specific differentiation antigens such as tyrosinase[6–11]; and (3) neoepitopes generated by point mutations in ubiquitously expressed genes, such as CDK4, β-catenin and MUM-1/L33-B (Refs 12–14). The variety and distinct nature of the identified TAAs suggests that CTLs can monitor genetic changes. Furthermore, they suggest that the MHC class I system provides an enormous diversity in the presentation of potential antigenic peptides, since at least six different MHC class I alleles may serve as restriction elements for human antitumor-directed CTL responses[15].

Although the identification of T-cell-defined TAAs has provided targets for T-cell-based immunotherapy, several prerequisites have to be met for the use of such strategies. These include the efficient processing and presentation of MHC class I-restricted antigenic peptides recognized by T cells, the generation and efficient activation of these T cells, and their subsequent homing to the site of malignancy.

The potential clinical significance of structural and functional abnormalities of MHC class I antigens expressed by malignant cells has rekindled interest in their characterization. It has been known for some time that malignant transformation is often linked to an increase in the rate of somatic mutations, providing tumor cells with mechanisms for escape from CTL-mediated immune surveillance. Loss of MHC class I allospecificities (either complete, locus-specific or allelic) has been identified in human malignant cells[16–18], and this is associated both with resistance of tumor cells to lysis by MHC class I-restricted CTLs and their enhanced sensitivity to lysis by natural killer (NK) cells[19–22].

The frequency of these abnormalities, their molecular mechanisms, and their functional and clinical significance have recently been reviewed[23,24]. The identification and characterization of components of the antigen-processing and presentation machinery has provided new tools for investigating how tumor cells evade immune surveillance. Current understanding of the physiology of this machinery will be reviewed below, followed by an analysis of the functional and structural defects of this pathway in malignant cells, and a discussion of the clinical impact of impaired MHC class I antigen processing and presentation in malignant diseases.

MHC class I antigen presentation

The classical MHC class I antigen pathway is operative in almost all cells capable of presenting peptides derived from endogenously synthesized proteins to CTLs (Refs 25–27) (Fig. 1). The multicatalytic proteasome complex, particularly the interferon γ (IFN-γ)-inducible subunits LMP-2 and LMP-7 (Refs 28–31), appears to be involved in the generation of antigenic peptide fragments (recently reviewed in Ref. 32). The generated peptides are shuttled into the endoplasmic reticulum (ER) by a nucleotide triphosphate (NTP)-dependent heterodimeric complex composed of the transporters associated with antigen processing – TAP1 and TAP2 (Refs 33–35). Apart from a preference for peptides with a distinct length (8–10 amino acids), the TAP heterocomplex selects peptides according to their affinities, this being influenced by the three N-terminal and the C-terminal amino acid peptide residues[36–40]. As with LMP-2 and LMP-7, the expression of TAP1 and TAP2 can be induced by IFN-γ stimulation[41], and the coordinate upregulation of these molecules is facilitated by common promoter elements[42]. A second mechanism of processing antigenic peptides may also occur: this TAP-independent pathway results from the proteolysis of hydrophobic signal peptide domains of proteins translocated into the ER (Refs 43, 44).

TAP is physically associated wth MHC class I molecules and mediates the loading of peptides into the MHC class I binding cleft[45]. Such peptides may either bind directly to MHC class I molecules or use a 'shuttle' protein. A potential candidate for such a chaperone function is the 48 kDa glycoprotein tapasin[46]. Finally, the trimeric complex comprising the MHC class I heavy chain, β_2-microglobulin and peptide reaches the cell surface via the trans-Golgi complex and presents epitopes to clonotypic T-cell receptors (TCRs)[47–49].

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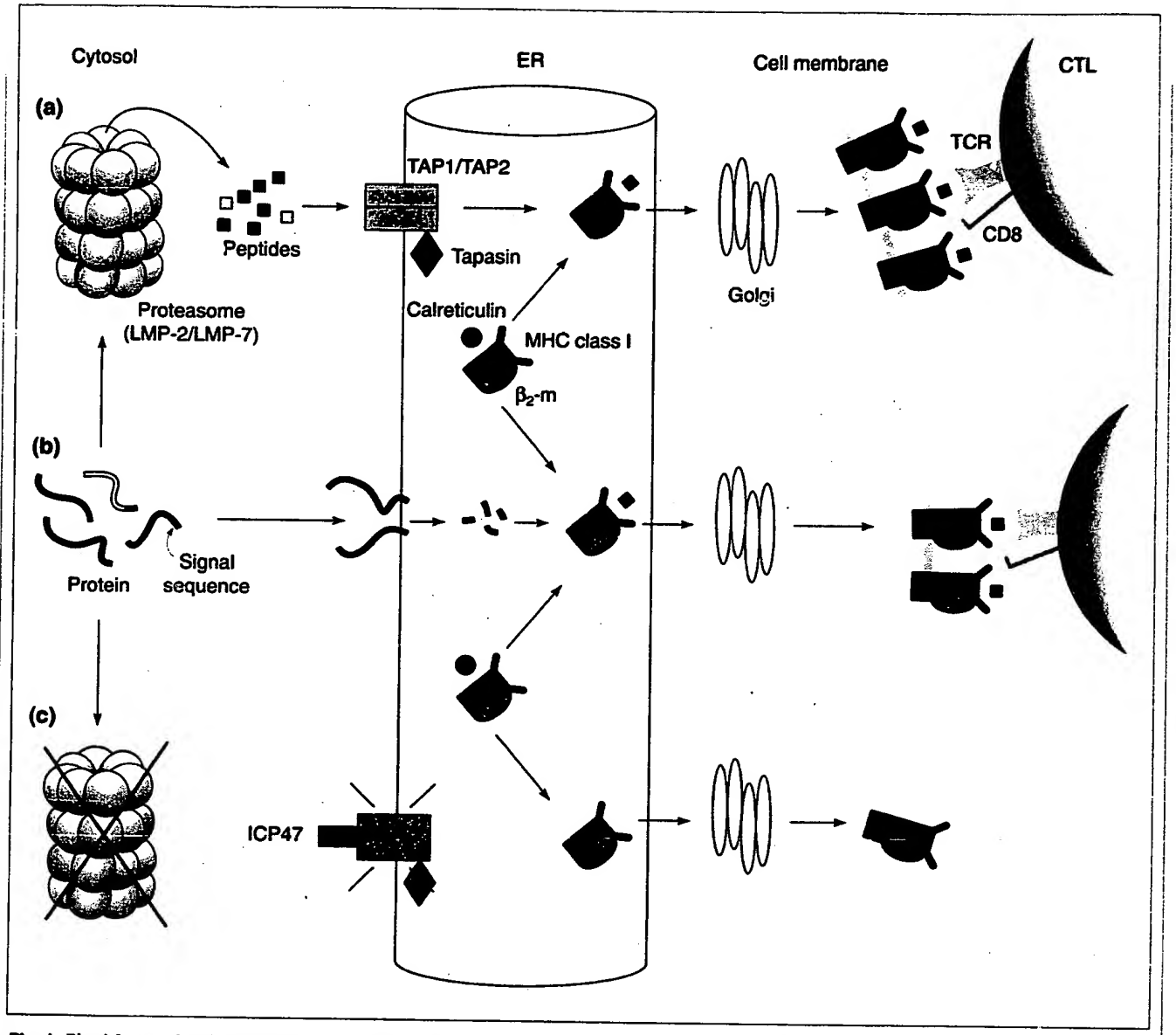


Fig. 1. *Physiology and pathophysiology of the classical MHC class I antigen presentation pathway. (a) In the TAP-dependent pathway, peptides cleaved by the proteasome subunits LMP-2 and LMP-7 are transported via the TAP heterocomplex into the ER, where they assemble with MHC class I chains and β_2-m to form the trimeric MHC class I complex. The interaction between TAP and MHC class I molecules occurs via the recently identified 48 kDa glycoprotein tapasin, which is capable of binding to TAP as well as to MHC class I/calreticulin complexes. Calreticulin functions as a molecular chaperone that determines whether MHC class I antigens are exported from the ER. (b) In the TAP-independent pathway, proteolysis of signal sequences in the ER provides peptides that are capable of binding the human MHC molecule HLA-A2. (c) Defects in the antigen-processing machinery resulting from LMP and/or TAP deficiencies result in reduced cell-surface expression of MHC class I antigen. As one example of this, the herpes simplex virus protein ICP47 blocks presentation of viral peptides by MHC class I antigens via binding to TAP and preventing peptide translocation into the ER. Abbreviations: β_2-m, β_2-microglobulin; CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; MHC, major histocompatibility complex; TCR, T-cell receptor.*

TAP is turned off in human tumors

The cell-surface expression of MHC class I antigens is reduced or lost in a variety of human tumors; these changes may represent one of several strategies utilized by tumor cells to evade CTL-mediated recognition and elimination[23,24]. For instance, LMP-2, LMP-7, TAP1 and/or TAP2 losses or dysfunction(s) have been shown in studies using tumor cell lines cultured *in vitro*[50], in antigen-processing mutants[51] and in surgically removed malignant lesions[52] (Fig. 1).

Defects in the ability to process and present antigenic peptides to $CD8^+$ CTLs were first described in small-cell lung carcinoma (SCLC) cells[50]. Restifo and co-workers tested human cell lines derived from solid tumors of different histotypes for antigen processing and presentation. Individual cell lines were infected with a recombinant vaccinia (Vac) virus to express the mouse MHC class I $H\text{-}2K^d$ molecule transiently, followed by evaluation of Vac-specific CTL recognition. Three SCLC lines that failed to transcribe genes encoding LMP-2,

Table 1. Downregulation of components of the antigen-processing and [illegible] in cultured human cell lines of different histological origin

Tumor type	LMP-2	LMP-7	TAP1	TAP2	Refs
Small-cell lung carcinoma	3/3	3/3	3/3	3/3	50
Burkitt's lymphoma	1/4	0/4	ND	ND	53
Hepatocellular carcinoma	ND	ND	2/7	1/7	54
Melanoma	2/9	2/9	5/9	5/9	55, 86
Prostate carcinoma	ND	ND	ND	2/5	56
Renal cell carcinoma	12/12	12/12	12/12	12/12	57, 58

[illegible] ND, not determined; RT-PCR, reverse transcriptase polymerase chain reaction.
[illegible] negative cell lines / number of tested cell lines.
[illegible] by RT-PCR.
[illegible] by [illegible] analysis.

LMP-7, TAP1 and TAP2 displayed low levels of transfected MHC class I antigens and were resistant to CTL-mediated lysis. Comparable results were obtained both in long-term-established and in freshly isolated Burkitt's lymphoma (BL) cell lines, some of which expressed low levels of endogenous MHC class I antigens on the cell surface due to defects in the antigen presentation machinery[53]. This abnormality, which has also been found in a hepatocellular carcinoma cell line[54], results in heterogeneous expression levels of TAP1 and TAP2 protein, reflecting an uncoordinated regulation of TAP1 and TAP2 expression. A similar mechanism, namely LMP-2, LMP-7, TAP1 and TAP2 downregulation, appears to underlie the reduced expression of MHC class I antigen in melanoma cells when compared with autologous Epstein–Barr virus (EBV)-transformed B cells (Ref. 55; B. Seliger, unpublished). By contrast, a different phenotype has been described in human prostate carcinoma cell lines, some of which display high cell-surface expression of MHC class I despite low levels of TAP2 mRNA (Ref. 56). These data are summarized in Table 1.

Analysis of primary renal cell carcinoma (RCC) cell lines revealed a heterogeneous pattern of LMP and/or TAP expression[57,58]. Genes encoding these proteins are downregulated both at the mRNA and protein level, although to a different extent in the individual cell lines. These findings suggest that abnormalities of the antigen-processing machinery in RCC cell lines occur at the transcriptional level. Furthermore, the downregulation and functional abnormalities of LMP and TAP were found to be more pronounced in RCC cell lines originated from metastatic lesions compared with RCC cell lines originated from primary tumor cells, whereas no defect was detected in autologous epithelial cells. These data suggest that abnormalities in the antigen-processing machinery are associated with malignant transformation and disease progression in patients with RCC.

Human tumors are frequently devoid of TAP in situ

Expression of the antigen-processing machinery has been analyzed in only a limited number of surgically removed tumor specimens of different histology. Representative immunohistochemical staining patterns are shown in Fig. 2. It is likely that such studies will be extended to a large panel of tumors since the reactivity of the available anti-LMP-2, anti-LMP-7, anti-TAP1 and anti-TAP2 xenosera with formalin-fixed and paraffin-embedded tissue sections facilitates the evaluation of banks of biopsy material stored in many laboratories[55].

The frequency of TAP1 downregulation varies significantly in different tumor types – ranging from 14% in primary colorectal carcinomas to 49% in primary human papillomavirus 16 (HPV-16)$^+$ cervical carcinomas (Fig. 2, Table 2). In the latter lesions, TAP1 downregulation was associated with suppression of MHC class I antigen expression[52]. Similar conclusions have been derived from immunohistochemical staining of surgically removed breast and colon carcinoma lesions[59,60]. In both malignancies, the frequency of downregulation of components of the antigen-processing machinery is higher in metastases than in primary lesions. In non-small-cell lung carcinoma, loss of the expression of the antigen-processing machinery is a frequent event. However, no correlation was found in this

disease between MHC class I antigen and TAP1 loss and the histological type, degree of differentiation, tumor stage, nodal stage or patients' survival[61].

Molecular lesions of the antigen-processing machinery in malignant cells

Translocation, loss of coding sequences or point mutations can potentially result in a loss of TAP function. However, no information is available on the molecular mechanisms underlying LMP-2, LMP-7 and TAP2 downregulation in malignant cells. Indeed, only one molecular lesion causing loss of TAP1 function without affecting its expression has been described: in one SCLC cell line, a point mutation near the adenosine triphosphate (ATP)-binding site of TAP1 results in the synthesis of a protein that cannot transport peptides[62].

Defects in the antigen-processing machinery might also result from alterations in the regulatory elements that control LMP and TAP transcription, as suggested by the restoration of the expression of these genes in malignant cells following incubation with cytokines (discussed below). An additional potential mechanism for functional deficiencies of the TAP complex is suggested by the functional characterization of the herpes simplex virus protein ICP47 in infected cells. This protein efficiently blocks peptide transport by forming a complex with TAP (Refs 63, 64). Therefore, one might speculate that malignant transformation of cells may be associated with the appearance of TAP inhibitors.

Altered CTL recognition of TAP-deficient tumor cells

It is not known whether downregulation of LMP-2 and LMP-7 in malignant cells causes any functional abnormalities. Such a possibility is suggested by the reduction in the repertoire of presented peptides and in the efficiency of their processing in murine cells that have decreased LMP-2 and LMP-7 expression[65]. Furthermore, it has been convincingly shown that defects in the peptide transport caused by TAP downregulation impair the assembly of MHC class I molecules in the ER, resulting in their low expression and stability on cell membranes. Thus, TAP defects provide malignant cells with a mechanism for escaping from CTL-mediated recognition. However, TAP abnormalities can also enhance susceptibility of target cells to lysis by NK cells[66]. The latter phenotype can be reversed by restoring TAP function by transfer of the gene encoding TAP, suggesting that efficient translocation of peptides from the cytosol into the ER is a prerequisite for abrogation of NK-cell-mediated recognition[66].

As mentioned earlier, some antigenic epitopes access the ER in a TAP-independent manner, and these would continue to be presented by tumor cells regardless of TAP deficiency. Such a pathway is utilized by the melanoma-associated antigen tyrosinase: one of two T-cell epitopes recognized by tyrosinase-specific HLA-A2-restricted CTLs is derived from the putative tyrosinase leader sequence, which thereby represents a potential TAP-independent T-cell epitope[67]. This may prevent melanoma cells from escaping T-cell responses through loss of the peptide transporters.

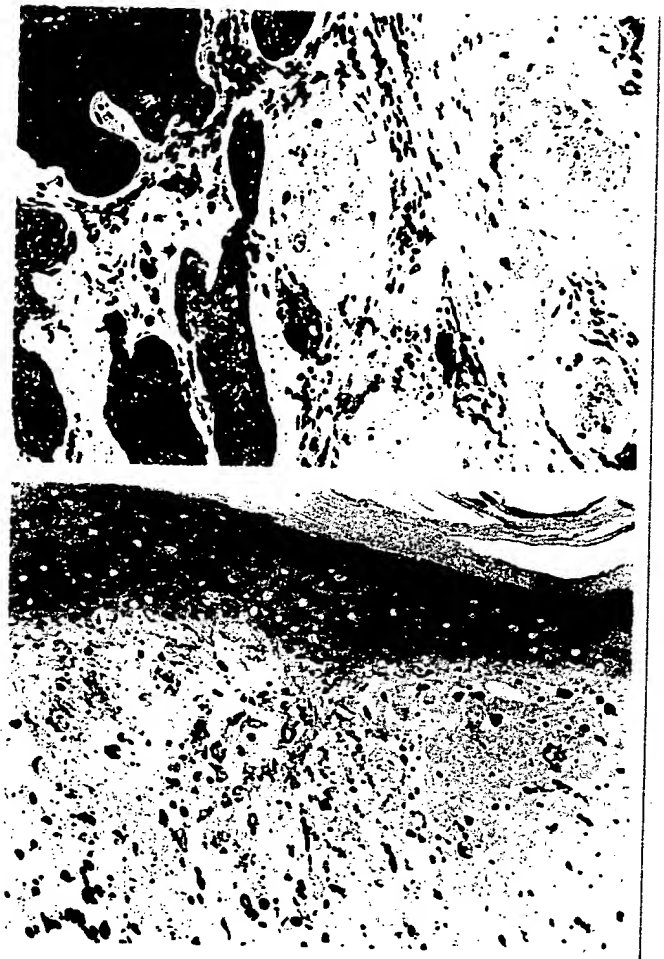
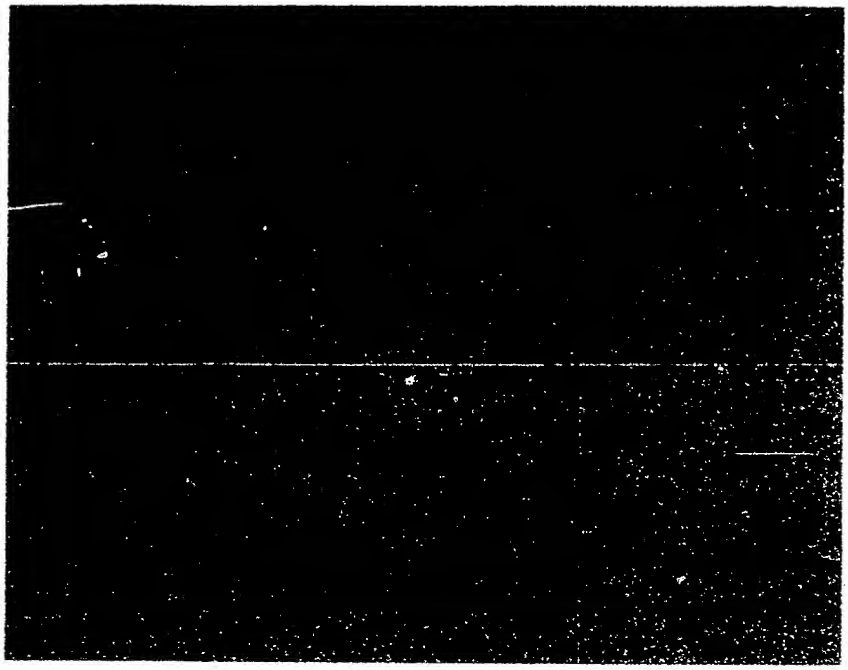


Fig. 2. *Immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections using purified rabbit anti-human TAP1 antibodies. (a) Cervical cancer: TAP1 staining is localized in the cytoplasm of some neoplastic areas (brown staining), whereas adjacent areas are TAP1⁻. (b) Melanoma lesions: TAP1 staining shows positive cytoplasmic staining of keratinocytes (light red), whereas melanoma cells are TAP⁻. Magnification = ×100.*

CTL epitopes may not be generally predictable from the protein sequence. Thus, a naturally processed tyrosinase peptide is modified by post-translational conversion of an asparagine residue to an aspartic acid residue, resulting in the attachment of carbohydrate side-chains. Although this alteration affects recognition by clonotypic TCRs, thereby enhancing the susceptibility to lysis, it does not change the binding affinity of the peptide for MHC class I molecules[63]. Whether the transport of such modified peptides into the ER is TAP dependent remains to be determined.

Recognition of TAP-defective tumor cells by other effector cells

TAP-deficient tumor cells exhibiting MHC class I downregulation may provide targets for immune effector cells other than $CD8^+$ CTLs. Indeed, the increase both of $CD4^+$ or $CD8^+$ αβ T cells and γδ



T cells in TAP2-deficient individuals suggests that the immune system may implement immune surveillance mechanisms that utilize TAP-independent restriction elements. These include MHC class II antigens[68], nonpolymorphic CD1 molecules[69], minor histocompatibility antigens such as TL (Refs 70, 71) and heat shock proteins (HSPs)[72]. Thus, melanoma-associated MHC class II antigens serve as restriction elements for melanoma-specific T cells, some of which show CTL effector functions[73,74]. In addition, cell-surface-associated HSPs present peptides derived from idiotypes in a human B-cell lymphoma cell line to γδ T cells[75]. Furthermore, the report that human CD1 molecules present non-peptide antigens raises the possibility that these structures may play a role in cell-mediated immunity directed against human carcinomas[76,77].

Mucins are preferentially expressed on tumor cells and display alterations in their expression levels and degree of glycosylation in breast, colon and pancreatic carcinoma cells, and may represent alternative target structures for CTL recognition in TAP-deficient tumor cells[78]. A similar role may be played by other, as-yet-undefined, target structures recognized by Vδ1$^+$ T cells on epithelial tumor cells, including lung carcinoma[79], RCC (Ref. 80) and colorectal carcinoma[81].

Box 1. Potential requirements for immunotherapy of tumor cells

1 Carry out immunomonitoring of the tumor to determine:
- HLA phenotype
- TAP phenotype
- Cytokine inducibility of TAP

2 Choose mode of immunotherapy:
- TAP$^+$ HLA-A2$^+$ tumor: CTL-based immunotherapy using A2 peptides
- TAP$^+$ HLA-A2$^-$ tumor: CTL-based immunotherapy using non-A2 peptides
- TAP$^-$ HLA-A2$^+$ tumor: CTL-based immunotherapy using signal peptide domains
- TAP$^-$ HLA-A2$^-$ tumor: non-CTL-based immunotherapy using alternate effector cells such as NK cells or γδ T cells

Abbreviations: CTL, cytotoxic T lymphocyte; NK, natural killer.

Functional effects of TAP restoration

Several proinflammatory cytokines, such as IFNs, are potent inducers of MHC class I antigens, LMP-2/7 and TAP1/2, although with different kinetics in endothelial, melanoma and RCC cell lines[41,57,58,82]. The IFN-γ-induced expression and function of TAP can lead to enhanced tumor-specific, MHC class I-restricted CTL recognition of melanoma, SCLC, prostate carcinoma and RCC (Refs 50, 56, 57, 83) (Table 3). These data suggest (1) that IFN-γ-mediated restoration of the antigen-processing machinery may be sufficient to regain CTL recognition of tumor cells in some patients; and (2) that patients with MHC class I downregulation on tumor cells due to LMP and/or TAP deficiencies may benefit from cytokine treatment.

An additional therapeutic strategy is suggested by the enhancement of MHC class I expression and the partial restoration of the peptide transport rate in RCC cell lines transfected with the wild-type gene encoding TAP1 (Ref. 84). In contrast to results obtained in a mouse model, the restoration of function does not appear to reflect the activity of TAP1 homodimers, but does reflect that of complexes resulting from the association of transfected TAP1 with endogenous TAP2 (Ref. 85).

Clinical significance of LMP and/or TAP abnormalities

LMP and/or TAP abnormalities are more frequent in metastases than in primary lesions. If this finding reflects a fortuitous event associated with the higher rate of genetic alterations in metastases due to genetic instability, then LMP and/or TAP loss may represent an epiphenomenon rather than a specific event in disease progression. Alternatively, the increased frequency of LMP and/or TAP abnormalities in metastases may reflect the immunoselection during clonal evolution of tumor cells exhibiting a growth advantage due to escape from recognition by MHC class I-restricted tumor-specific CTLs. This possibility is supported by the recently described loss of TAP1 and melanoma-associated MART-1/Melan-A antigen in a patient with lethal recurrent melanoma[86]. Although the tumors had responded well to immunotherapy, the patient succumbed to

metastatic disease. Detailed analysis revealed that the tumor escaped immune control because of a sequential loss of targets for CTLs and a downregulation of TAP expression[86]. These findings provide circumstantial evidence that TAP1 loss may indeed represent one major mechanism for immune escape of malignant cells. It can also be speculated that a vigorous MHC class I-restricted CTL response may aid immunoselection *in vivo* and participates in the generation of 'immune escape' variants exhibiting MHC class I deficiencies due to aberrant TAP expression.

Conclusions

Malignant transformation of human cells may be associated with downregulation or loss of MHC class I antigen expression. This may be caused by structural alterations or dysregulation of either the MHC class I molecules or the components of the antigen-processing and presentation machinery. Cells expressing this phenotype may escape CTL-mediated immune surveillance since, at least *in vitro*, they display a marked reduction in their susceptibility to lysis by MHC class I-restricted, tumor-specific CTLs. Research into abnormalities of the antigen-processing machinery in malignant cells is in its infancy. Only a small number of tumors have been investigated for LMP and/or TAP deficiencies and, within each tumor type, only a few surgically removed lesions have been analyzed. The limited information available from these studies raises a number of questions, which are considered below.

LMP-2 and/or LMP-7 are frequently downregulated in malignant cells; do these defects reduce the repertoire of TAAs presented or the efficiency of their presentation? Furthermore, the frequency of TAP downregulation displays marked differences in various types of neoplasias; do these differences reflect a differential role of these abnormalities in the pathogenesis and/or clinical course of distinct types of tumors? The higher frequency of TAP downregulation in metastases compared with primary lesions in some malignancies argues in favor of a role of abnormalities in tumor progression. However, there are conflicting results concerning the correlation of these defects with histopathological characteristics of lesions and/or the clinical course of diseases. Such an association has been identified in patients with melanoma, in whom tumor regression may be mediated by MHC class I-restricted CTLs, but not in those with lung or colon carcinoma. In the latter, recognition of tumor cells by CTLs has not been convincingly demonstrated. Whether the association between TAP downregulation and histopathological parameters found in only some of the malignancies reflects differences in the characteristics of the patients investigated, in the methodology and reagents used, or in the role of CTL-mediated immune responses in various types of malignant diseases, remains to be elucidated.

In a panel of melanoma cell lines, the lack of translation of genes encoding LMP-2, LMP-7, TAP1 and TAP2 has been found more frequently than the lack of transcription. Is this a general phenomenon? If this turns out to be the case, screening malignant cells at the mRNA level for defects in the antigen-processing machinery is likely to underestimate their frequency.

The transfer of genes encoding cytokines or TAP has been found to restore, at least partially, the function of the antigen-processing machinery in malignant cells *in vitro*. Are these approaches effective *in vivo*? Should the administration of cytokines be included in the protocols of specific immunotherapy in patients with malignant diseases? Several practical questions need to be addressed, such as the dosage, duration, tolerance and response rates of cytokine treatment.

Finally, several issues regarding potential immunotherapeutic strategies need to be addressed: does a vigorous antitumor-directed CTL immune response favor immunoselection of a TAP-deficient population of tumor cells *in vivo*? Does selection of patients on the basis of TAP expression in malignant lesions improve clinical response rates in T-cell-based immunotherapies? Box 1 lists potential strategies for immunotherapy of tumor cells. Further analysis of these issues will contribute to the assessment of the clinical significance of LMP and TAP defects in tumors, and particularly their role in the interaction of malignant cells with the immune system of the host.

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Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition

Salvatore Valitutti and Antonio Lanzavecchia

Sensitivity of antigen detection by B cells correlates with high-affinity binding. This paradigm does not appear to hold for the T-cell receptor (TCR), which is able to bind its ligand – peptide in the context of major histocompatibility complex (MHC) – with low affinity. Here, Salvatore Valitutti and Antonio Lanzavecchia propose that the efficiency of T-cell antigen recognition is dependent upon optimal kinetics of the TCR–peptide–MHC interaction, allowing serial engagements and triggering of many TCRs by a few peptide–MHC complexes.

Interaction of the T-cell receptor (TCR) with specific peptide bound to major histocompatibility complex (MHC) molecules triggers an elaborate cascade of signaling events that ultimately leads to cell-cycle progression, interleukin (IL) production and even cell death[1–3]. The earliest detectable event is the phosphorylation by *src*-family kinases of tyrosine residues present within specific immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of the CD3 and ζ-chain components of the TCR complex. This leads to recruitment and activation of ZAP-70, followed by a variety of enzymes and adaptors, and ultimately to the activation of different signal transduction pathways.

T-cell antigen recognition is exquisitely sensitive. Indeed, T helper (Th) cells can proliferate and produce cytokines in response to antigen-presenting cells (APCs) displaying as few as 50–100 specific peptide–MHC complexes[4,5]. Even more strikingly, a single peptide–MHC class I complex appears to be sufficient for triggering cytotoxicity[6].

Given the high sensitivity of T cells, the finding that TCRs bind specific peptide–MHC complexes with low affinity came as a great surprise. Indeed, using separate approaches and different TCRs, the affinity of this interaction was estimated to be much lower than that of antibodies for antigens, ranging between 10^{-4} and 10^{-7} M (Refs 7–10). In particular, the TCR–peptide–MHC interaction is characterized by a very high off-rate, with half-lifes ranging from four seconds to a few minutes[9–11]. These results appeared to be paradoxical: it was difficult to envisage how low-affinity TCRs might allow T cells to recognize with high specificity a low number of peptide–MHC complexes[12].

Since the TCR and its cognate ligand interact as integral membrane proteins on two opposing cells, it is not possible to describe their interaction in the terms used to describe antigen–antibody interaction in solution. For instance, the formation of the complex will be influenced by the adhesion between T cells and APCs, and the stability of the complex may be modulated by accessory

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Presentation of Endogenous Peptides to MHC Class I-Restricted Cytotoxic T Lymphocytes in Transport Deletion Mutant T2 Cells

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Abstract. The ability of minigene-encoded viral peptide epitopes to be presented by class I molecules in the absence of MHC-encoded transporters has been evaluated in mutant T2 cells. These cells have a large deletion in the class II MHC region that includes the known transporter protein for antigenic peptides and proteasome genes and they are defective in presenting viral epitopes to CTL. T2 cells that express minigenes encoding the influenza virus matrix peptide 58-66 (GILGFVFTL) and two HTLV 1 Tax peptides 11-19 (LLFGYPVYV) and 12-19 were lysed by HLA-A2-restricted peptide-specific CTL. Minigene expression of a HLA-A2-restricted HIV reverse transcriptase peptide 476-484 (ILKEPVHGV) with three charged residues sensitized T2 cells poorly for lysis by HIV-specific CTL unless the peptide was preceded by an endoplasmic reticulum translocation signal sequence. Expression of an influenza virus nucleoprotein peptide 383-391 (SRYWAIRTR) with three charged arginine residues did sensitize HLA-B27$^+$ T2 cells for lysis by peptide-specific CTL. These and other results with endogenously expressed peptide analogs in which hydrophobic and charged amino acids were interchanged demonstrate that antigenic peptides can be translocated from the cytoplasm into the class I Ag presentation pathway independent of MHC-encoded transporters; and that peptide hydrophobicity appears not to be a major determinant in selecting peptides for this alternate pathway. *Journal of Immunology,* 1993, 150: 1763.

Recently genes have been identified within the MHC of mice, rats, and man encoding presumed ATP-dependent TAP[2] (1–6) and proteasome subunits (5–8). These findings strongly suggest that proteasomes mediate degradation of cytoplasmic proteins into peptides which are then translocated by TAP into an exocytic intracellular compartment (probably the ER; see Ref. 9) where they assemble into a trimolecular complex with class I H chain and β_2-microglobulin. Additional support for the role of TAP in peptide translocation derives from experiments in which the mutant phenotype (i.e., susceptibility to CTL-mediated lysis after virus infection) of cell lines .134 and RMA-S which are defective in one of the transporter protein subunits was reversed after transfection

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[2] Abbreviations used in this paper: TAP, transporter proteins for antigenic peptide; ER, endoplasmic reticulum NP, nucleoprotein; RT, reverse transcriptase.

with cDNA coding for wild type subunit (2, 3, 10, 11). However, recent reports suggest the existence of another peptide translocation mechanism for class I presentation, independent of the presumed ATP-dependent TAP. First, in vitro experiments showed that microsomal vesicles prepared from T2 cells (lacking both TAP) were not deficient in translocation of antigenic peptides and that peptide translocation into microsomal vesicles of wild type and mutant T2 cells did not require ATP (12–14). (For a discussion of T2 cells and phenotypically normal parental T1 cells, see *Materials and Methods*). Second, RMA-S cells (with one defective TAP and presumably with a functional Ag degradation machinery) infected with influenza virus or with vesicular stomatitis virus were recognized by virus-specific CTL, albeit less efficiently than wild type cells (15, 16).

To investigate the mechanism of peptide transport into the class I presentation pathway independent of protein degradation, we have studied endogenous peptide loading of HLA-A2 with a stable transfection system using the episomal plasmid p8901 encoding minigene DNA for the influenza virus matrix protein-derived peptide 57-68 (KGILGFVFTLTV). T1 cells expressing peptide 57-68 were lysed by peptide-specific CTL, whereas 57-68 transfected T2 cells were not; however, T2 cells expressing peptide 57-68 fused to an endoplasmic reticulum signal sequence (from the adenovirus E3/19-kDa protein) were recognized by peptide-specific CTL (17). These results suggested that translocation of peptide 57-68 did not occur in the absence of the TAP gene products in T2 cells and that this defect was reversed by providing an alternate route of peptide transport into the ER.

Recently we observed that a synthetic nonapeptide derived from the influenza virus matrix protein (58-66: GILGFVFTL) is 100- to 1000-fold more active than peptide 57-68 in sensitizing HLA-$A2^+$ cells to lysis, and that endogenous expression of peptide 58-66 sensitized phenotypically wild type HLA-$A2^+$ cells to lysis by peptide-specific CTL (18). Octa- and nonapeptides are associated with MHC class I molecules at the cell surface (19, 20) and these peptides might be presented to CTL more efficiently than longer peptides such as 57-68. It is possible therefore that the susceptibility of virus-infected RMA-S cells to lysis requires the generation of these optimal peptides from viral proteins in the cytoplasm and their uptake into the endoplasmic reticulum by a TAP-independent mechanism. It is also possible that T2 cells become susceptible to lysis if appropriate peptides are provided in the cytoplasm through the endogenous expression of minigenes. To test this experimentally, the optimal nonapeptide 58-66, three short HLA-A2-restricted peptides derived from the HTLV Tax protein and HIV RT, and an HLA-B27-restricted peptide derived from the influenza virus NP were expressed as minigenes in T1 and T2 cells and transfected cells were assayed for susceptibility to CTL-mediated lysis.

Materials and Methods

Cell lines

HMy.C1R cells (21) (referred to as C1R) (a kind gift from Dr. Peter Cresswell, Yale University, New Haven, CT) transfected with HLA-A2 (C1R:A2) and HLA-B27 (C1R:B27) have been previously described (22, 23). T1 cells (24) are a hybrid between the mutant cell line 721.174 (25) (which expresses HLA-A2 at reduced levels and has deleted most of the MHC class II region including the known TAP and proteasome genes) and the T lymphoblastoid cell line CEM that has a normal complement of class II genes (24). T2 cells were derived from T1 cells after the spontaneous loss of CEM-derived chromosome 6, and express cell surface HLA-A2 at 30 to 50% of the level of T1 cells and, after infection, fail to present viral Ag to CTL (24). T1 and T2 cells as well as T2 cells transfected with HLA-B27 (26) were kindly provided by Dr. Cresswell.

Construction of episomal vectors with minigene DNA

The expression plasmid p8901 was used to express minigenes. (We do not know for any of the minigene-derived peptides discussed in this manuscript to what extent the N-terminal initiating methionine was removed after synthesis in the cytoplasm (27) (see also *Discussion*). Therefore, endogenously generated peptides will be referred to as (met)57-68, (met)58-66, etc.). Plasmids expressing influenza virus matrix peptides 57-68 ((met)KGILGFVFTLTV) and 58-66 ((met)GILGFVFTL), and plasmids expressing these peptides fused to the adenovirus E3/19-kDa protein ER translocation sequence ((met)RYMILGLLALAAVCSAA), have been described (17, 18, 28). DNA coding for analogs of the matrix peptides ((met)57-68K57A and (met)58-66G58K), for peptides 12-19 ((met)LFGYPVYV) and 11-19 ((met)LLFGYPVYV) from HTLV I Tax protein (29), for peptide 476-484 ((met)ILKEPVHGV) from HIV RT (30, 31) and peptide 383-391 ((met)SRYWAIRTR) from influenza virus nucleoprotein (23) were synthesized in a similar way using the appropriate overlapping oligonucleotides, PCR, and *Bam*HI and *Not*I digestion. Plasmid p8901 with the insert coding for peptide 57-68 was digested with *Bam*HI and *Not*I, the 57-68 DNA insert removed by gel filtration, and analog DNA ligated into this site.

Transfection of episomal minigene expression vectors

T1 and T2 cells were transfected as described (17). These cells will be referred to as T1(ctrl), T1(matrix57-68), T1(matrix58-66), T1(Tax12-19), etc. to indicate T1 cells expressing control plasmid p8901 without minigene DNA and p8901 plasmids coding for influenza virus matrix protein-derived peptides (met)57-68 and (met)58-66 and HTLV I Tax protein derived peptide (met)12-19, etc.

Cytotoxic T cell lines and assays

HLA-A2-restricted CTL specific for influenza virus type A matrix peptide 58-66 and HTLV I Tax 11-19 were generated and characterized as previously described (29). The generation of HLA-A2-restricted HIV RT peptide 476-484-specific CTL and HLA-B27-restricted influenza virus NP peptide 383-391-specific CTL has also been described (23, 30, 31). CTL assays were performed as described (17, 18, 28) and the results are reported as mean percent specific lysis of triplicate determinations. For anti-class I antibody blocking experiments, 50 μl of mAb W6/32 (32) was incubated with target cells (5×10^3/50 μl) at 37°C for 20 min. Effector cells were then added (10^4/50 μl) and the assay incubated at 37°C until the level of lysis in the absence of antibody reached 30 to 60% specific lysis.

Cell surface expression of class I molecules

Cell surface expression of class I molecules was analyzed by indirect immunofluorescence using either monoclonal antibodies BB7.2 (32), CR11-351 (33), or MA2.1 (34) for detection of HLA-A2 and ME1 (35) for detection of HLA-B27. Fluorescein-conjugated F(ab')2 fragment of goat anti-mouse IgG (Organon Teknika, West Chester, PA) was used as the detecting reagent. Cell surface fluorescence was analyzed on a FACScan flow cytometer (Becton Dickinson, Braintree, MA).

Cell sorting

C1R:A2(ctrl) and T2(matrix58-66) cells were cocultivated for 3 days, after which sterile sorting of these cells was performed on a FACStar Plus Flow Cytometer/Consort 40 (Beckton Dickinson, Mountain View, CA) using FITC-L243 (anti-HLA-DR4, Becton Dickinson) antibody (T2 cells are MHC class II$^-$). Upper and lower limits were set for right and left sorts independently and cells in the overlap region were not sorted. Upon completion, the two sorted populations were analyzed by flow cytometry and found to be pure.

Peptide hydrophobicity calculations

The average hydrophobicity (36) of peptides was calculated by the Helwheel program of PC/Gene (IntelliGenetics, Inc., Mountain View, CA).

Results

HLA-A2 and HLA-B27 levels on surface of T2 cells that express minigene-derived peptides

Cell surface expression of HLA-A2 molecules on T2 cells is 30 to 50% of that found on T1 cells (25). T2 cells transfected with genomic HLA-B27 DNA express roughly

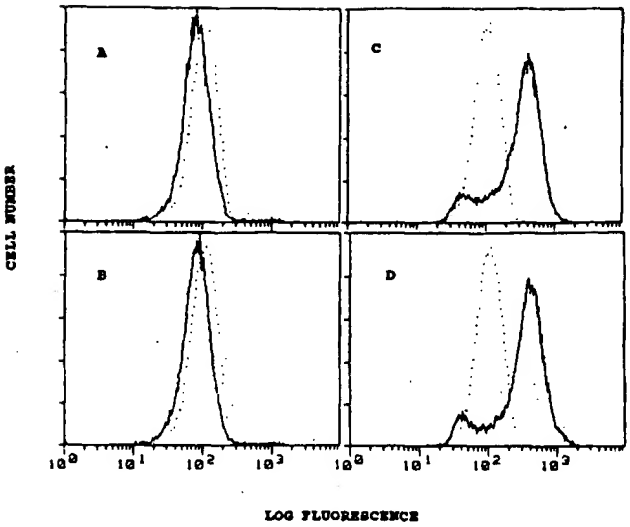

FIGURE 1. Cell surface expression of HLA-A2 on T2 cells transfected with vector p8901 with minigene DNA encoding peptides Tax (met)11-19 and Tax (met)12-19 with and without the signal sequence. T2 cells expressing Tax (met)12-19 (*A*), Tax (met)11-19 (*B*), SigTax 12-19 (*C*), and SigTax 11-19 (*D*) were assayed for levels of cell surface expression of HLA-A2 by indirect immunofluorescence with the anti-HLA-A2 antibody BB7.2 and fluorescein-conjugated goat anti-mouse IgG and analyzed by flow cytometry. *Solid lines* are the profiles of T2 cells expressing the indicated peptides and the *dotted lines* are profiles of T2 cells transfected with the p8901 vector alone. Similar profiles were obtained with cells stained with the anti-HLA-A2 antibodies CR11-351 and MA2.1 as well as the class I-specific antibody W6/32 (data not shown).

50-fold less surface HLA-B27 compared to HLA-B27-transfected HMy.C1R cells (data not shown). Transfection of T2 cells with vector p8901 with minigenes encoding the HLA-A2-restricted peptides (met)58-66 and (met)57-68 derived from the influenza A virus matrix protein and peptide (met)476-484 derived from HIV polymerase as well as T2 cells transfected with the p8901 vector alone did not result in increased levels of cell surface expression of HLA-A2 (data not shown; for peptide nomenclature, see *Materials and Methods*). Similar results were obtained when these peptides were preceded by an ER translocation sequence derived from the adenovirus E3/19-kDa protein. Furthermore, expression in HLA-B27$^+$ T2 cells of minigenes encoding the HLA-B27-restricted influenza NP peptide (met)383-391 with or without the signal sequence did not increase surface levels of HLA-B27 molecules (data not shown). Expression of HTLV I Tax peptides (met)11-19 and (met)12-19 did not increase surface HLA-A2 expression either. However, HLA-A2 levels at the surface of T2 cells that expressed the HTLV I Tax peptides (met)11-19 and (met)12-19 with the ER signal sequence were increased significantly (Fig. 1).

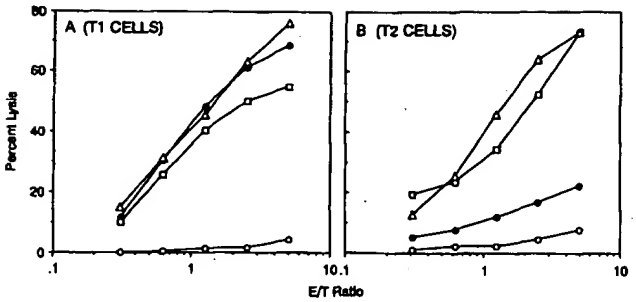

FIGURE 2. Lysis of T1 (Fig. 1*A*) and T2 (Fig. 1*B*) cells stably transfected with episomal plasmid p8901 with minigene DNA coding for the influenza virus matrix protein derived peptides 58-66 ((met)GILGFVFTL) with (△) or without (□) the Adenovirus E3/19K signal sequence, coding for peptide 57-68 ((met)KGILGFVFTLTV) (●) or transfected with p8901 without minigene DNA (○).

Table I
T2(matrix58–66) cell supernatants do not sensitize HLA-A2⁺ cells

Targets	E:T	% Lysis
C1R:A2(ctrl)[a]	2:5	7.8
	1:25	0.6
C1R:A2(ctrl) plus peptide 58–66[b]	2:5	44.2
	1:25	32.3
Cocultivated C1R:A2(p8901)[c]	2:5	7.2
	1:25	0
T2(matrix58–66)	2:5	41.7
	1:25	21.7
Cocultivated T2(matrix58–66)[c]	2:5	37.3
	1:25	28.1

[a] C1R:A2 cells with plasmid p8901 without minigene DNA.
[b] At 10^{-3} μM.
[c] C1R:A2(ctrl) and T2(matrix58–66) cells were cocultivated for 3 days. Afterward cells were sorted into HLA-DR⁻ T2(matrix58–66) and HLA-DR⁺ C1R:A2(p8901) cells and assayed for susceptibility to lysis by matrix peptide-specific CTL at 2 E:T ratios. C1R:A2(ctrl) and T2(matrix58–66) cells were also assayed without prior cocultivation.

Minigene expression of influenza virus matrix peptide 58-66 sensitizes T1 and T2 cells

It was shown previously that T1 cells, but not T2 cells, expressing the influenza matrix peptide (met)57-68 (KGILGFVFTLTV) were recognized by peptide-specific CTL; and that expression of peptide 57-68 fused to the adenovirus E3/19-kDa endoplasmic reticulum translocation signal sequence did sensitize T2 cells for lysis (17). To extend this work, the nonamer 58-66 (GILGFVFTL), which is the optimal matrix protein-derived peptide for HLA-A2 binding and CTL recognition (18, 37), was expressed in T1 and T2 cells with and without the E3/19-kDa translocation signal sequence. The results in Figure 2 demonstrate that transfected T1 and T2 cells (referred to as T1(matrix58-66) and T2(matrix58-66) cells) were susceptible to lysis by peptide-specific CTL regardless of whether peptide 58-66 was preceded by the signal sequence.

Previous experiments established that CTL recognition of cells expressing endogenous peptides with or without a signal sequence was not due to release of peptides from the cells and reassociation from the medium with cell surface class I molecules (17, 18, 28). We established that the same was true for T2(matrix58-66) cells. First, supernatants from a 5-day-old culture of T2(matrix58-66) cells were unable to sensitize T2(ctrl) cells or C1R:A2(ctrl) cells for lysis by peptide-specific CTL (data not shown). Second, C1R:A2-(ctrl) cells that were cocultured with T2(matrix58-66) cells at a 1:1 ratio for 3 days and positively selected by FACS sorting using HLA-DR-specific antibody L243 were not susceptible to lysis by peptide-specific CTL unless they were preincubated with synthetic peptide 58-66 (Table I).

Endogenous expression of peptides derived from HTLV I Tax protein sensitizes T1 and T2 cells

The nonstructural Tax protein of HTLV I induces HLA-A2-restricted CTL that recognize target cells preincubated with either the synthetic octapeptide Tax 12-19 (LFGYPVYV) or the nonapeptide 11-19 (LLFGYPVYV) but that do not recognize HLA-A2⁺ targets sensitized with the influenza matrix peptide 58-66 (29). Minigenes encoding these two Tax peptides with or without the signal sequence were transfected into T1 and T2 cells and were assayed for recognition by HLA-A2-restricted Tax-specific CTL lines. The results (Table II) demonstrate that T2(Tax11-19) and T2(Tax12-19) cells were lysed to a comparable extent as T1(Tax11-19) and T1(Tax12-19) cells. T1 and T2 transfectants with the signal peptide fused to both Tax11-19 and 12-19 were recognized to the same extent (data not shown). CTL recognition of these minigene transfectants was peptide specific because Tax peptide-specific CTL did not lyse T2(matrix58-66) cells and matrix peptide-specific CTL did not lyse Tax minigene transfectants (Table II).

Recognition of T2 cells expressing Tax peptides with the signal peptide might be more efficient compared to T2 cells expressing Tax peptides without the signal sequence because the former transfectants express higher levels of cell surface HLA-A2 (Fig. 1). Therefore, experiments were performed to determine whether anti-class I antibody W6/32 had a differential inhibitory effect on recognition and lysis of Tax peptide-transfected T2 cells with or without the signal sequence. The results in Figure 3 demonstrate that even though the T2(Tax11-19) and T2(Tax12-19) cells were lysed to a comparable extent as T2(signalTax11-19) and T2-(signalTax12-19) cells, the Tax minigene transfectants without the signal sequence were more susceptible to anti-class I antibody blocking. These results are consistent with the view that the signal sequence has enabled more of the Tax peptide to enter the class I Ag presentation pathway and produce a greater number of peptides-HLA-A2 complexes that are recognized by CTL.

Table II
HTLV I Tax minigene products in T2 cells are recognized by peptide-specific CTL

Transfectants	% Specific Lysis by Effector Cells[a]			
	Anti-tax		Anti-matrix	
	2.5:1	0.6:1	2.5:1	0.6:1
T1(Tax12–19)	80	48	11	8
T2(Tax12–19)	70	40	0	2
T1(Tax11–19)	100	74	5	0
T2(Tax11–19)	91	77	25	4
T2(Matrix58–66)	3	10	69	55

[a] CTL specific for HTLV I Tax and influenza virus matrix peptides (lines RSCD8#7 and 193.5, respectively) were generated as described (11). Amino acid composition of peptides was as follows: 58–66: (met)GILGFVFTL; Tax 11–19: (met)LLFGYPVYV; Tax 12–19: (met)LFGYPVYV.

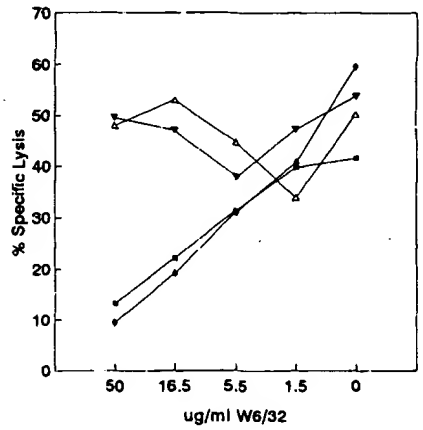

FIGURE 3. Differential inhibition of lysis of T2 transfectants with anti-class I antibody. T2 cells expressing peptides SignalTax12-19 (▼), SignalTax11-19 (△), Tax(met)12-19 (□), or Tax(met)11-19 (◆) were preincubated for 20 min with the indicated concentrations of W6/32 antibody and then assayed for susceptibility to lysis by the anti-Tax specific CTL clone RSCD8#7. Results are shown for E:T of 2:1. No lysis was observed on T2 cells transfected with the p8901 vector alone (data not shown).

CTL recognition of T2 cells expressing HLA-A2-restricted peptide derived from HIV RT

The ability of T2 cells to translocate the influenza matrix peptide (met)58-66 and the Tax peptides (met)12-19 and (met)11-19 in the absence of MHC-encoded transporters may be due to the hydrophobicity of these peptides. This may explain why endogenous expression of peptide (met)-57-68 with a positively charged lysine in position 57 did not sensitize T2 cells for lysis.

To test this hypothesis, we assessed the ability of T2 cells to translocate and present a viral peptide epitope with a number of charged residues. The HIV RT-derived peptide (RT476-484: ILKEPVHGV) (31) contains two positively charged residues (Lys and His) and one negatively charged residue (Glu). The results in Table III demonstrate that T2-

Table III
Lysis of T1 and T2 cells expressing HIV RT peptide (met)476–484[a]

Transfectants	% Specific Lysis	
	10:1	5:1
T1(ctrl)[b]	0.5	−1.0
T1(RT476–484)	85.0	91.6
T1(SigRT476–484)	93.8	86.3
T1(p8901) + RT476–484[c]	87.7	74.1
T2(ctrl)[b]	−0.2	0.1
T2(RT476–484)	22.6	19.8
T2(SigRT476–484)	89.4	86.0
T2(p8901) + RT476–484[c]	96.2	95.4

[a] Amino acids: ILKEPVHGV.
[b] Control cells transfected with vector p8901 without minigene DNA.
[c] Synthetic peptide RT476–484 was added at 10 μM.

(RT476-484) cells were lysed significantly less (22.6%) by peptide-specific CTL (31), compared to T1(RT476-484) (85% lysis). Both T2 and T1 cells expressing RT476-484 preceded by the adenovirus E3/19-kDa translocation signal sequence were lysed efficiently.

CTL recognition of T2 cells expressing HLA-B27-restricted peptide derived from influenza virus nucleoprotein

The effect of charged residues on peptide translocation in T2 cells was investigated further by expressing the HLA-B27-restricted influenza virus nucleoprotein-derived peptide NP 383-391 (SRYWAIRTR) that contains three positively charged Arg residues. This nonamer is optimal for binding to HLA-B27 and for CTL recognition (23). Vector p8901 with minigene DNA encoding this peptide with or without the signal sequence were transfected into HLA-B27$^+$ T2 cells or into phenotypically wild type HLA-B27$^+$ HMY.C1R cells (26). The results in Figure 4 demonstrate that NP(met)383-391-expressing T2 cells are recognized by NP 383-391-specific HLA-B27-restricted CTL (see Ref. 23) but that recognition is not as effective as that of HLA-B27$^+$ HMY.C1R cells that express NP(met)383-391. Similar results were obtained in two additional independent experiments (data not shown). The more efficient lysis of the latter cells does not appear to reflect a greater intrinsic susceptibility to lysis because T2 cells that express NP-(met)383-391 preceded by the signal sequence were lysed to a comparable extent as the HLA-B27$^+$ HMY.C1R cells expressing the same fused peptide (Fig. 4). Lysis of the HLA-B27-transfected T2 cells expressing NP(met)383-391 was specific because they were not lysed by HLA-A2-restricted matrix peptide-specific CTL (data not shown). Thus, NP peptide (met)383-391 can be translocated and presented in T2 cells, but the efficiency of presentation is enhanced by the presence of the signal peptide.

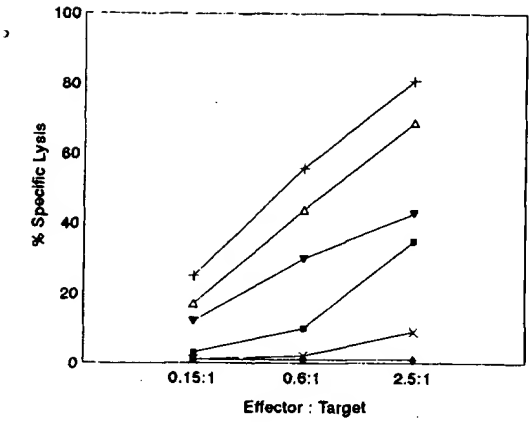

FIGURE 4. Lysis of C1R:B27 and T2 cells stably transfected with p8901 with minigene DNA coding for NP peptide (met)-383-391 (SRYWAIRTR) with or without the E3/19kDa signal sequence. +, T2(signalNP383-391); ■, T2(NP383-391); X, T2(p8901); △, C1R:B27(signalNP383-391); ▼, C1R:B27-(NP383-391); ◆, C1R:B27(p8901).

CTL recognition of minigene transfectants that express modified influenza virus matrix protein-derived peptides

The possible role of charged residues in CTL recognition of T2 cells was investigated further by testing sensitization of T2 cells expressing an analog of peptide 57-68 with the hydrophobic residue Ala replacing charged Lys at position 57 ((met)AGILGFVFTLTV; referred to as (met)57-68K57A) were tested for susceptibility to lysis. We showed previously that wild type HLA-A2$^+$HMy.C1R cells that express this minigene construct were recognized by peptide-specific CTL.[3] The results in Figure 5*A* demonstrate that T2(met)57-68K57A cells were nearly as susceptible to lysis as T2(met)58-66 and that, as shown before, T2 cells expressing the more charged peptide (met)57-68 were not lysed. In the second experiment, the opposite substitution was made in peptide 58-66 (Gly in position 58 was replaced by a charged Lys). T2 cells expressing analog ((met)58-66G58K) were nearly as sensitive to lysis as T2 cells expressing peptide (met)58-66 (Fig. 5*B*).

Thus the presence or absence of a single charged residue in matrix protein-derived peptides 57-68 or 58-66 is not a major determinant in allowing presentation of endogenously generated peptides in T2 cells.

Peptide hydrophobicity and ability to present in T2 cells

The average hydrophobicity values of the antigenic peptides tested in this report are listed in Table IV (larger values indicate increased hydrophobicity), along with a measure of the ability of these peptides to be presented in T2 cells.

[3] Submitted for publication.

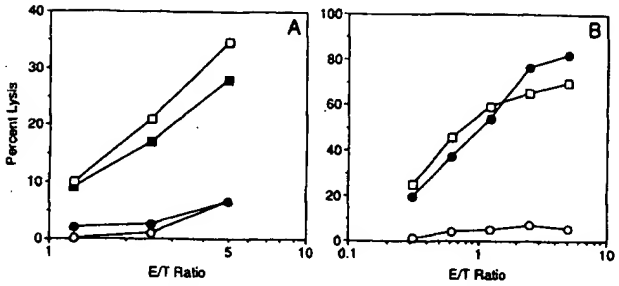

FIGURE 5. Lysis of T2 cells stably transfected with p8901 with minigene DNA coding for modified influenza virus matrix peptides. *A*, 57-68 ((met)KGILGFVFTLTV) (●), 57-68K57A ((met)AGILGFVFTLTV) (■), 58-66 ((met)GILGFVFTL) (□), without minigene DNA (○). *B*, 58-66 ((met)GILGFVFTL) (●), 58-66G58K ((met)KILGFVFTL) (□), without minigene DNA (○).

Table IV
Comparison of peptide hydrophobicity and ability to present in T2 cells

Peptide	Sequence	Hydrophobicity[a]	Tap Indep. Presentation[b]
M57–68	KGILGFVFTLTV	0.61	0.10
M57–68K57A	AGILGFVFTLTV	0.79	0.64
M58–66	GILGFVFTL	0.86	0.87
M58–66G58K	KILGFVFTL	0.65	0.72
TAX12–19	LFGYPVYV	0.69	0.87
TAX11–19	LLFGYPVYV	0.73	0.91
RT476–484	ILKEPVHGV	0.28	0.26
NP383–391	SRYWAIRTR	−0.53	0.56[c]

[a] See *Materials and Methods.*
[b] TAP independent presentation = lysis of T2 transfected cells/lysis of T1 transfected cells. Lysis was determined at limiting E:T ratios and corrected for lysis of T1 and T2 cells transfected with p8901 without minigene DNA.
[c] In the case of NP383–391, lysis in transfected T2 cells was expressed relative to that in C1R:B27 cells.

The matrix protein-derived peptide 57-68 that is presented poorly has an average hydrophobicity value (0.61) that is almost identical to that of matrix peptide analog 58-66G58K (0.65) and Tax 12-19 (0.69) that are presented efficiently. Hydrophobicity of the poorly presenting HIV RT-derived peptide 476-484 (0.28) is significantly less than that of the matrix and Tax protein-derived peptides (≥0.61). The least hydrophobic peptide is NP383-391 (−0.53) which is presented, albeit less efficiently. This peptide is presented to an extent comparable to that of the matrix peptide analog 57-68K57A that is the second most hydrophobic peptide investigated (0.79). This analysis did not allow us to establish a clear correlation between average peptide hydrophobicity and ability to present in T2 cells.

Discussion

Our experimental results show that mutant T2 and wild type T1 cells present three different short (8 and 9 amino acids long) HLA-A2 and one -B27-restricted viral peptide

epitopes that are generated in the cytoplasm of these cells (Fig. 2 and 4, Table II). A fourth HLA-A2 restricted nonameric epitope derived from HIV RT (amino acids 476-484) was presented inefficiently in T2 cells but efficiently in T1 cells (Table III). (Previously we reported that a 12 amino acid long matrix protein-derived peptide 57-68 was not presented in T2 cells (17)). Because T2 cells lack both MHC-encoded transporter proteins, this selective peptide translocation into the intracellular exocytic compartment has to occur by a TAP-independent mechanism. This process needs not to be very efficient because our experimental read out, susceptibility to lysis, is very sensitive; only a few hundred peptide-class I complexes are needed for efficient lysis (39).

We do not know whether translocation by this alternate pathway occurs by mechanism(s) similar to those used by proteins containing a signal sequence or by passive diffusion uptake. Signal sequences are diverse in amino acid composition but they share a 7-13 amino acid long hydrophobic core sequence located within a 15-30 residue region (40, 41); and the region at the N-terminus of the core sequence contains positively charged residues (40). It is possible that the three positively charged arginine residues in the influenza virus nucleoprotein-derived peptide 383-391 (SRYWAIRTR) facilitate ER translocation. Although we were unable to establish for the other peptides a clear correlation between hydrophobicity and ability to present in T2 cells (see Table IV) we cannot rule out that hydrophobicity influences peptide translocation by the TAP-independent pathway.

The selectivity of presentation of the various peptides in this study by class I molecules in T2 cells need not reflect differences in uptake into the ER. Presentation may be influenced by decreased peptide concentrations in the cytoplasm caused by lower rates of synthesis or increased turnover; or by differential affinities of peptides for the class I molecules. We have not been able to quantitate endogenously generated peptides. Peptide degradation is unlikely to be controlled by the N-end rules (42) and therefore the identity of specific N-terminal amino acids provides little information to predict peptide stability. Another variable that is difficult to verify and that may affect peptide stability, translocation, and/or class I restricted presentation concerns the removal of the N-terminal methionine from expressed peptides. Methionine removal is influenced largely by the nature of the penultimate N-terminal amino acid (27). Both the HTLV I Tax protein-derived peptides that sensitize T2 cells and the HIV RT-derived peptide that poorly sensitizes T2 cells have Leu at the N-terminus (Leu prevents methionine removal); the nonsensitizing matrix protein-derived peptide 57-68 and the sensitizing analog 58-66G58K have Lys (prevents methionine removal) at the N-terminus; and Gly and Ala (residues that generally facilitate methionine removal) are at the N-terminus of the sensitizing peptides 58-66 and 57-68K58A (Fig. 5). The issue of the affinity of different peptides for class I molecules needs to be addressed by in vitro experiments to determine the kinetics of peptide binding.

T2 cells transfected with minigene DNA coding for the HTLV I Tax protein-derived octameric and nonameric peptides preceded by the adenovirus E3/19-kDa signal sequence expressed significantly more surface HLA-A2 (Fig. 1). Sequencing of endogenous peptides indicated that a significant proportion of HLA-A2 molecules on T2 cells bound peptides derived from signal sequences (43, 44). This raises the possibility that peptides derived from the E3/19-kDa signal sequence and not those from the Tax proteins were bound to HLA-A2 in T2(signalTax12-19) and T2(signalTax11-19) minigene transfectants. Endogenously expressed influenza virus matrix protein and HIV RT-derived peptides fused to the adenovirus E3/19-kDa signal peptide failed to increase surface expression of HLA-A2. This suggests that the increased surface HLA-A2 molecules were occupied by Tax protein-derived peptides, possibly because of more efficient binding and assembly of the class I molecules with these peptides.

Whatever the parameters that control peptide transport and presentation, the results clearly show that, independent of the TAP transport system, peptides can gain access to the intracellular exocytic compartment where class I molecules and peptides associate. These findings support those reported by others who showed that virus-derived peptides were translocated in vitro into microsomes of T2 cells (12) and that, both in wild type and mutant cells, this did not require ATP (12, 14). Furthermore, it indicates that when appropriate peptides are generated in virus-infected cells, they can enter the ER via this alternate pathway and associate with class I molecules (15, 16).

Physiologically, the TAP-independent pathway may be important in the early stages of virus infection. Expression of TAP genes may be relatively poor and TAP-independent peptide transport may play an important role in the initiation of virus-specific cell-mediated immune responses. As the infection proceeds, IFN-γ is released, up-regulates the expression of proteins that are encoded in the MHC region including TAP (45) and more efficient peptide, translocation follows.

Note Added In Proof. Recently, a HLA-A2 restricted epitope from the HIV gag protein corresponding to amino acids 77-85 (SLYNTVATL; hydrophobicity value 0.33) was identified (Johnson et al., manuscript in preparation). Endogenous expression of this epitope and a 12-amino acid sequence containing this epitope (amino acids 74-85 (ELRSLYNTVATL; with a hydrophobicity value of 0.06) in T1 and T2 cells resulted in efficient lysis by peptide specific CTL. This represents an other example of peptides with

polar and charged residues that enter the intracellular exocytic compartment via a TAP-independent mechanism.

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